MECHANISTIC IMPACT OF THE SWEDISH APP-MUTATION AND CASPASE-3 CLEAVED C–TERMINAL PRESENILIN FRAGMENT IN THE NEUROTOXIC EFFECTS OF BETA-AMYLOID

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MESCHANISTISCHE AUSWIRKUNGEN DER SCHWEDISCHEN APP-MUTATION UND DES CASPASE-3 GESPALTENEN C-TERMINALEN PRESENILIN FRAGMENTS AUF DIE NEUROTOXISCHEN EFFEKTE VON BETA AMYLOID

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1. Introduction

1.1 THE IMPACT OF ALZHEIMER'S DISEASE, A CHALLENGE TO SCIENCE AND SOCIETY

In 1907, Alois Alzheimer, a German Psychiatrist, described at a meeting in Tübingen the symptoms as well as the neuropathological features of a strange degenerative disease of the brain cortex (Alzheimer 1907). His original patient, a woman referred to as Auguste D. in his report, suffered from several symptoms like progressive memory impairment; disordered cognitive function; altered behaviour including paranoia, delusions, loss of social appropriateness and a progressive decline in language function.

Neither he nor his audience could expect the impact of this disease for the 21th century. Due to an improved medical care, life expectancy increased dramatically within the last century. This resulted in a burgeoning number of individuals achieving the age at which neurodegenerative disorders become evident. Today, Alzheimer's disease (AD) is recognized as a major public health problem in developed nations and the most common cause of dementia among people aged 65 and older. The impact of AD for the major public health acquires a growing importance for our society:

- According to the American National Institut of Aging, AD affects probably 20–30 million people worldwide.
- An estimated 10 percent of Americans over the age of 65 and half of those over age 85 have AD.
- Currently, more than four million Americans currently suffer from this disease, and the number is projected to balloon to 10-15 million over the next several decades.
- AD is after heart disease and cancer the third most expensive disease to treat in the USA., costing society close to $100 billion annually.
- In Germany actually about 1.000.000 people have AD. This number is expected to duplicate within the next decade as a result of an adjustment of the age pyramid.

Besides the financial aspect for the health care, of course there is the dramatic impact of AD for the individual families. The patients are helpless and require full-time health care. Families, friends and caregivers struggle with great emotional and physical stress to cope with the mental and physical changes of the concerned family member.

For many decades after Alzheimer's original description, little progress in defining the pathogenesis of AD occurred. Only in the past decade the knowledge of its causes and mechanisms has grown enormously. Thanks to modern biochemical, genetic, cell biological and transgenic modeling studies, today the composition and molecular origin of the amyloid plaques and neurofibrillary tangles, the two hallmarks of AD, have been described. Nevertheless, the mechanisms leading to neurodegeneration, the formation of plaques and tangles is still not completely understood. Despite an intensive search for therapeutic intervention, no drug has proven effective in combating this devastating neurodegenerative disease. The answers to many questions regarding the understanding of the progression of AD pathology are still need missing and imply a big challenge for researchers worldwide.

1.2 PATHOLOGICAL CHANGES IN AD BRAIN

1.2.1. Changes in Brain

The clinical appearence of AD is a progressive degeneration of neurons in the cerebral cortex. In severe AD stages the brain weight differs notably, compared to non-AD affected same aged brains. According to Braak et al. (1994) the pathological changes in the AD brain can be divided into the following stages:

1. Preclinical stage

   The first brain changes probably start 10 to 20 years before any visible signs or symptoms appear. The first brain region affected by plaques and tangles is the entorhinal cortex, localized near to the hippocampus. Than it proceeds to the hippocampus. Affected regions begin to atrophy.

2. Mild Stage

   The cerebral cortex is affected. Symptoms like memory loss, mood or personality changes are typical signs for mild AD.

3. Moderate Stage

   AD damage has spread to areas of the cerebral cortex that control language, reasoning, sensory processing, and conscious thought. The symptoms of this stage include increasing memory loss and confusion or difficulty with language. In addition, the patients can suffer from hallucinations, delusions, paranoia or from motoric problems.

4. Severe Stage

   In this stage, plaques and tangles are widespread throughout the brain, and areas of the brain have further atrophied. Patients are completely dependent on others for care and suffer from weight loss, seizures, infections and lack of bladder and bowel control. Since patients ultimately become immobile and suffer from respiratory difficulties, most patients die from other illnesses like pneumonia.

The defining neuropathological lesion of AD are extracellular amyloid plaques containing beta amyloid peptide (Aß) and intracellular neurofibrillary tangles containing abnormally phosphorylated tau protein aggregated into filaments. These histological abnormalities are associated with loss of synaptic density and neuronal death.

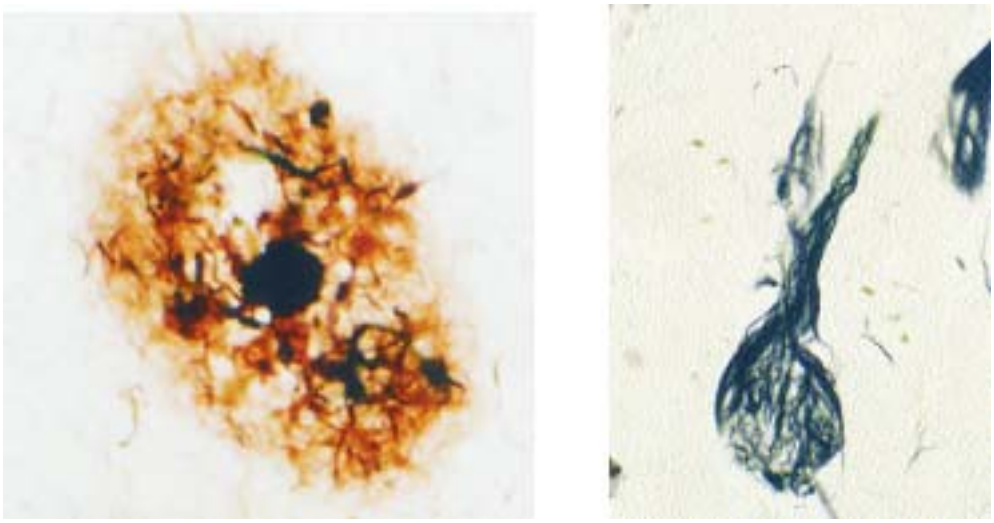

Figure 1.1. Pathological hallmarks in AD brain: a plaque, mainly consisting of Aß (left). The dense amyloid core is surrounded by an amyloid halo. Methamine staining. Neurofibrillary tangles (right) are formed by dead neuronal cell bodies due to cytoskeletal changes, for instance hyperphosphorylation of tau protein. Gallyas staining (pictures taken from Bogdanovic 2000).

1.2.2 Amyloid plaques

Neuritic amyloid plaques are roughly spherical, extracellular deposits of beta amyloid protein (Aß) fibrils intimately surrounded by dystrophic axons and dendrites, activated microglia, and reactive astrocytes. Most of the fibrillar Aß found in the neuritic plaques, is the species ending at amino acid 42, the slightly longer, more hydrophobic form that is particularly prone to aggregation (Jarrett 1993). However, the Aß 40 colocalizes with Aß 42 in the plaque. The cross-sectional diameter of neuritic plaques in microscopic brain sections varies widely from

10 to 120 µm. In addition to Aß, other proteins like ApoE, or lysosomal proteins are found in plaques (Atwood 2002). The occurrence of lipids rafts and cholesterol was also described (Burns 2003).

The fibrillar amyloid plaques are invariably accompanied by many "diffuse" (pre-amyloid) plaques in the same brain regions. These do not consist of fibrilla extracellular deposits of A*β*-immunoreactive granular material that generally lack amyloid fibrils and are associated to very few or no dystrophic neurites. In most AD cases, the number of diffuse plaques clearly exceeds that of the neuritic plaques. Diffuse plaques appear to represent the earliest light-microscopically detectable lesion in AD brains. They seem to predate fibrillar neuritic plaques, as judged by their occurrence in cognitively normal, late middle-aged and elderly healthy individuals (Dickson 1995), as well as by their development in teenagers with Down's syndrome, long before the latter individuals develop the neuritic plaques and neurofibrillary tangles typical of AD (Selkoe 2002).

1.2.3 Neurofibrillary tangles

Neurofibirllary tangles (NFT) correspond to the aggregation of abnormally phosphorylated Tau proteins into paired helical filaments. Tau protein, a neuronal microtubule-associated protein, is known for its role in the stabilization of microtubules, which is important for the generation and maintenance of neurites (Cleveland 1977, Binder 1985). In AD, phosphorylated Tau accumulates in neurons, aggregates into paired helical filaments, and loses its microtubule-binding and stabilizing function, leading to the degeneration of neurons (Garcia 2001).

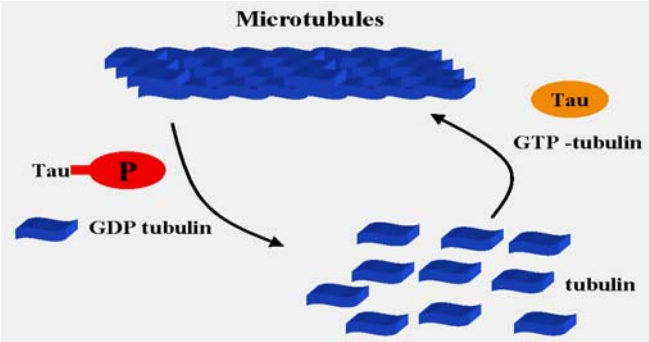

Fig. 1.2. Tau phosphorylation and microtubule polymerization. Polymerization of tubulin into microtubules is regulated by the state of phosphorylation of Tau proteins. Hypophosphorylated Tau induces tubulin polymerization and microtubule stabilization whereas hyperphosphorylated Tau lead to the depolymerization of microtubules into tubulin.

The phosphorylation of Tau is regulated by different kinases and phosphatases, but the exact mechanism leading to an enhanced Tau phosphorylation is still not clear. Tau has approximately 20 to 30 potential phosphorylation sites, many of which are putative targets of proline-directed serine/threonine kinases. Accumulating evidence suggests that two such kinases, glycogen synthase kinase-3 (GSK-3ß) and cyclin-dependent kinase-5 (cdk5), are major tau kinases (Cruz 2003). Other involved kinases described are p42/44 MAP kinase (Mandelkow 1999) and the c-Jun N-terminal kinase (JNK) (Reynolds 1997; Bue´e-Scherrer 2002).

1.3 GENETICS OF ALZHEIMER'S DISEASE

1.3.1 Causative genes of inherited forms of AD

AD is usually classified according to its age of onset. When the disease occurs before 65 years of age it is called early-onset ("presenile") AD form, while late-onset ("senile") AD occurs in subjects over 65 years of age. About 7 percent of early-onset cases are familial, with an autosomal dominant pattern of inheritance and a nearly 100 % penetrance (Campion 1999). Thus, familial forms of early-onset Alzheimer's disease (FAD) are rare (less than 1 % of all AD cases); however, their importance extends far beyond their frequency, because they have allowed researchers to identify some of the critical pathogenetic pathways of the disease.
Due to family linkage studies and DNA sequencing, three genes have been identified whose mutations cause early-onset FAD. These genes encode for the amyloid precursor protein (*APP, see 1.4.1*) on chromosome 21, the presenilin 1 (*PS1, see 1.4.2*) on chromosome 14 and the presenilin 2 (*PS2*) localized on chromosome 1 (Goate 1991, St George-Hyslop 1994).

1.3.2 Apolipoprotein E

In addition to the causative genes of early onset FAD, a genetic "risk factor" for late-onset FAD, the apolipoprotein E (Apoε) on chromosome 19, has been identified (Saunders et al., 1993). Three variants of the gene and the protein are found in human populations and result from changes in single amino acids in ApoE (referred to as the Apoε2, ε3 and ε4 alleles). Whereas the Apoε3 and Apoε2 alleles have no or even beneficial effects, possession of the Apoε4 allele, especially homozygosity for Apoε4, augments the risk for AD. The effects of

Apoε4 allele is "dose-dependent". The mean age of onset decreases from 84 to 68 years with increasing numbers of Apoε4 alleles (Corder 1993).

Genetic Factors for AD						
	Chromosome	Gene	Identified Mutations	Begin of disease	Penetrance	Phenotype
EO FAD	21	APP	8	45-64	100%	Aß↑
	14	Presenilin 1	>120	28-55	100%	Aß↑
	1	Presenilin 2	8	40-75	100%	Aß↑
EO AD				< 65		
LO FAD	19	Apolipo-protein E	Poly-morph. ε 2, ε 3, ε 4	61-65	Riskfactor ε 4	Density of Aß plaques↑
LO AD				> 65		

TABLE 1.1. Confirmed genetic factors predisposing to Alzheimer's disease. To date more than 120 different mutations were identified in the PS1 gene leading to early onset (EO) FAD. Eight mutations were discovered in APP and PS2 gene (http://molgen-www.uia.ac.be/ADMutations). Mutations in these causative genes have a 100% penetrance leading to an enhanced Aß production. The Apolipoprotein E polymorphism is a confirmed risk factor for the late onset (LO) AD. Other genetic factors which taken alone are not sufficient to develop AD are discussed in 1.3.3.

How Apoε4 leads to an enhanced AD risk is still not clear. ApoE is a lipid carrier molecule involved in the transport and distribution of cholesterol and lipids from liver to all extra hepatic tissues via LDL-receptor mediated mechanism. ApoE is a plasma glycoprotein with a molecular mass of 34,2 kDa synthesized mainly by the liver, by both neurons and astrocytes in the brain, and also by other cell types including macrophages and monocytes. ApoE contains 299 amino acids, the amino terminal domain (residues 1–191) is a stable globular structure containing the receptor binding site, while the carboxy-terminal domain (residues 216–299) is helical, less stable, and contains the lipoprotein binding functions (Mahley 2000). In brain, ApoE is not involved in the transport of cholesterol to and from the brain but instead is involved in redistribution of cholesterol to different cellular and subcellular sites of neurons

needed for membrane remodelling during regeneration of neurites, axons and synapses (Kirsch 2002). ApoE is also involved in many other functions, like nerve regeneration, immunoregulation and activation of several lipolytic enzymes (Vancea 2000). Distinct binding properties of ApoE isoforms to Aß and Tau protein have suggested ways by which ApoE might mediate its action (Myers 2001). In particular, the Apoε4 isoform binds to Aß more rapidly than the Apoε3 isoform. Apoε4 associated with Aß forms novel monofibrils that precipitate into dense structures. Apoε4 does not bind to Tau protein *in vitro*, unlike Apoε2 and Apoε3. It is possible that the interaction between Apoε3 and Tau protein serves as protection against Tau phosphorylation and neurofibrillary tangle formation (Strittmatter 1994). An amyloid-scavenging role of ApoE has been proposed too. ApoE might regulate extracellular Aß through ApoE receptor-mediated internalisation of Aß via an endosomal/lysomal path (Beffert 1996). A role of ApoE during the development, maturation, and aging of the CNS was also reported (Nathan 1994), since ApoE promotes neuritic extension in an isoform-dependent manner (Apoε2 > Apoε3 >Apoε4).

1.3.3 Other genetic risk factors

Although age and the inheritance of predisposing genetic factors appear to play a major role, recent evidence suggests that the development and progression of AD could be subject to a wide variety of both environmental and genetic modifiers (Tanzi 2001). Several other genetic factors have been implicated in AD. In the majority of sporadic AD cases genetic factors may act as predisposing agents, without the force to induce the disease but able to increase the risk of disease above that of the general population. They probably interact with environmental factors or with other pathological or physiological conditions to exert their pathogenic effect.
The typical approach to evaluate genetic contribution to the risk for AD is analysing the frequency distribution in cases and controls of the allelic variants at polymorphic sites of the candidate genes. Most of these candidate genes identified in case-control association studies are proteolytic enzymes, plasma proteins, growth factors or membrane receptors, which may exist as different genetic variants. A lot of these genes are involved in APP processing and/or in the degradation and clearance of Aß. These genes are potential risk factor for AD, however, their significance needs to be confirmed by additional studies. Following candidate genes have been described.

- The gene of α2-Macroglobulin (α2M) on chromosome 12, a serum protease inhibitor also expressed in the brain, has been implicated in AD on the basis of its ability to mediate the clearance and degradation of Aß. α2M is also a component of senile plaques (Blacker 1998).
- Low density receptor-related Lipoprotein (LRP) gene is also localized on chromosome 12. It is the main ApoE receptor expressed in neurons (Rebeck 1995). It is responsible for the endocytosis of secreted amyloid precursor protein (Verpillat 2001) and it has been detected in senile plaques, dystrophic neurites and reactive astrocytes in AD brain (Kounnas 1995).
- Angiotensin converting enzyme (ACE) which catalyses the conversion of angiotensin I to angiotensin II, by cleaving the two carboxy-terminal amino acids from angiotensin. It is widely expressed in the endothelium of cerebral blood vessels and in neurons, where it contributes to the modulation of pressor response, neuro-humoral function, and behaviour (Wright 1992). A polymorphism at intron 16 of the *ACE* gene (chromosome 17), consisting in an insertion/deletion of a 287 base pairs sequence, has been found to be associated with AD susceptibility (Kehoe 1999). An increased frequency of the insertion allele was reported among patients with late-onset AD (Narain 2000).
- Insulin degrading enzyme (IDE) gene is located on chromosome 10. It codes for a 110 kDa extracellular thiol metalloprotease capable of degrading a number of peptides including insulin and Aß (Selkoe, 2001). It has been shown that IDE may be the protease responsible for the clearance of Aß (Vekrellis 2000, Bertram 2000) and the cytoplasmic fragment of APP following liberation of Aß (Edbauer 2002).
- Nitric oxide synthase (NOS3, chromosome 7) gene. There is growing evidence showing an involvement of nitric oxide (NO) in the AD pathology. The NOS3 endothelial product (eNOS) has a high concentration in hippocampal pyramidal neurons (Doyle 1997). Moreover, NO production by microglial cells, astrocytes and brain endothelium is enhanced in AD patients because of a strikingly increased expression of eNOS (De la Monte 1997). In addition, Aß has been reported to stimulate NO production in neurons harboring Presenilin 1- (Hashimoto 2002) and APP mutations (Keil 2004). Finally, there is a growing evidence of an involvement of NO in neuronal death in AD. Nitrosative stress caused by increased NO synthesis in the brain could be a pathogenic mechanism for AD (Eckert 2003). Dahiyat et al. (1999) hypothesized that polymorphic variants in the NOS3 gene might facilitate

degenerative changes. A common structural polymorphism,Glu/Asp, at codon 298 of NOS3, has been analysed and the homozygous Glu 298 genotype has been found to be significantly over-represented in late-onset AD patients compared with control individuals.

- c-Jun NH2 terminal kinase interacting protein-1 (JIP-1), which functions as a neuronal scaffold protein to allow signalling specificity. JIP-1 interacts with many cellular components including the LRP, kinesin and the Alzheimer's amyloid precursor protein. JIP-1 colocalized with JNK and phosphorylated tau in neurofibrillary tangles. A 499Asp>Glu polymorphism in the 5' regulatory region of the JIP-1 gene was decribed by Helbecque (2003) whereby the G allele was proposed to be a risk factor to the onset of AD.

1.4 MOLECULAR MECHANISMS OF ALZHEIMER'S DISEASE

1.4.1 The Amyloid-Precursor-Protein

Studies on amyloid precursor protein (APP) as genetic determinant of AD have begun in the middle 1980s with the observation that individuals with Down's syndrome invariably develop the clinical and neuropathological features of AD if they live over 30 years (Mann 1985). These data pointed to the involvement of chromosome 21 in AD and supported the theory that overexpression of a gene mapping on chromosome 21, present in an extra copy in Down's syndrome, could produce the AD phenotype. Later, the gene encoding for APP was the first gene identified as the leading cause of hereditary AD (Tanzi 1987, Goate 1991).

APP comprises a heterogeneous group of ubiquitously expressed polypeptides with molecular weights between 110 and 140 kDa. This heterogeneity arises from alternative splicing (yielding 3 major isoforms of 695, 751, and 770 residues) as well as by a variety of posttranslational modifications, including the addition of *N*- and *O*-linked sugars, sulfation, and phosphorylation (Selkoe 1994, Walter 1997). The APP splice forms containing 751 or 770 amino acids are widely expressed in nonneuronal cells throughout the body and also occur in neurons. However, neurons express even higher levels of the 695-residue isoform, which occurs at very low abundance in nonneuronal cells (Haass 1994).

The APP undergoes at least two proteolytic cleavages. One pathway involves the membrane-associated α-secretase, which cleaves APP within the Aß domain and secretes the

extracellular N-terminal of APP (soluble APPα, Hooper 1997). This way is not pathogenic (Selkoe 1994b). The other cleavage pathway, occurring in the endosomal–lysosomal compartment (Haas 1992), involves ß-secretase, which cuts between residues 671 and 672 of the APP, and yields the N-terminus of Aß peptide. In addition, APP can be processed by a third proteolytic cleavage, the γ-secretase, yielding the C-terminus of Aß peptide (see Fig.1. 3). The cleavage site of γ-secretase is of critical importance as it may generate peptides of different lengths. The 40 amino acid long Aß peptide is the most common form usually produced in the endosomal–lysosomal system by a cleavage at residue 712–713. A peptide of 42 amino acids (Aß42) in length is generated from a cleavage site after residue 714, which is thought to be more fibrillogenic and neurotoxic (Lorenzo 1994).

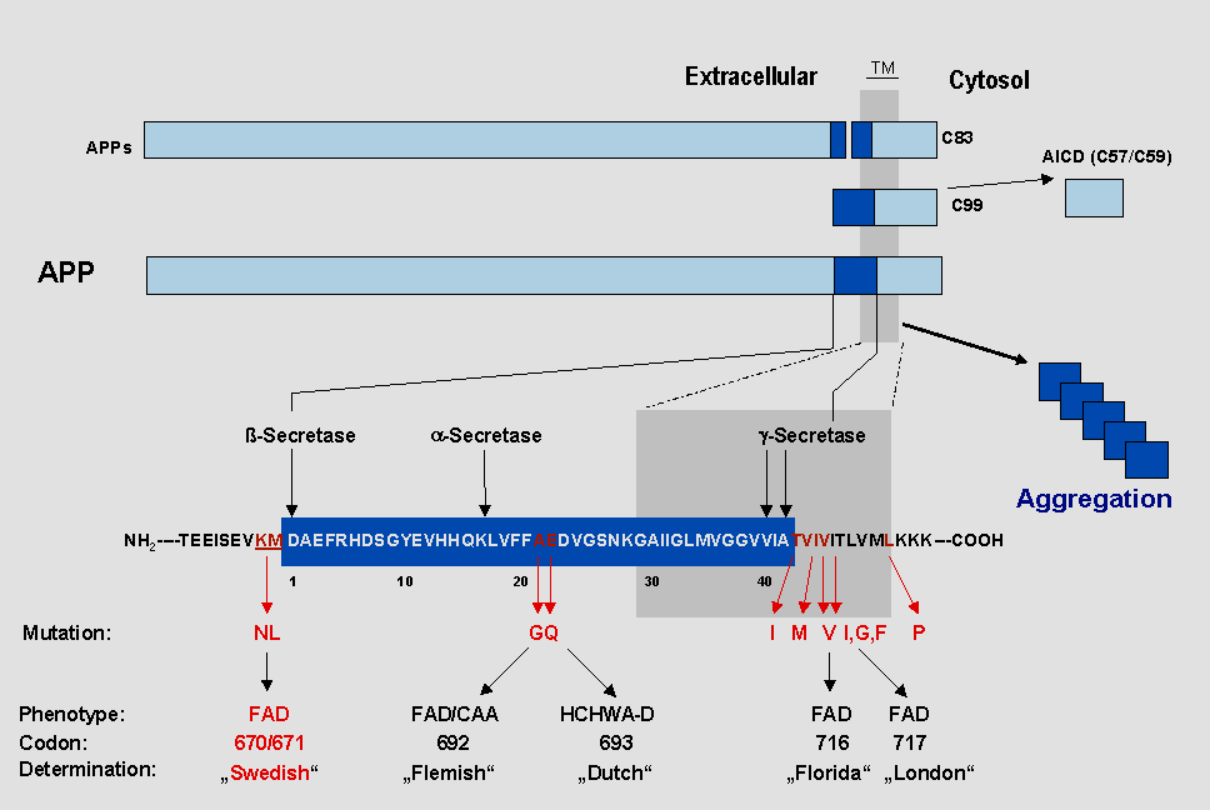

Figure 1.3. Schematic diagrams of the amyloid precursor protein (APP). The processing of APP by a protease designated as α-secretase leads to the secretion of a large soluble ectodomain of APP (APPs) and retention of a 83-residue COOH-terminal fragment in the membrane. Alternatively, cleavage of APP by ß-secretase produces a membrane-bound C-terminal fragment, C99 which is further cleaved by a γ-secretase leading to the release and secretion of pathogenic Aß-peptide and a C-terminal fragment designated as AICD (APP intracellular domain: C57/C59). Missense mutations in the APP-gene either interfere with α-secretase or enhance ß- or γ-secretase cleavage, resulting in an increased production of the toxic Aß peptide.

The function of APP is still poorly understood. *In vitro* studies suggest that secreted APPα can function as an autocrine factor by stimulating cell proliferation and cell adhesion and supporting nerve growth factor-induced neurite outgrowth of PC12 cells (Villa 2001). Other studies have implied a role for APP in signal transduction (Nishimoto 1993) or, in association with other proteins, e.g. Fe 65, in regulation of transcription (Cao 2001). Recent data indicate a pysiological function of APP, as a cargo receptor for the microtubule motor kinesin, in the anterograde transport of several transmembrane proteins (Kamal 2001, Verhey 2001).

APP is highly conserved in evolution, but the deletion of the APP gene in mice results in neither early mortality nor appreciable morbidity. This lack of vital consequence of APP deletion *in vivo* may result from expression of the homologous proteins the amyloid precursor-like proteins (APLPs) (Slunt 1994). Delineation of the precise functions of APP and its homologs *in vivo* awaits further study. No evidence has emerged that a fundamental cellular function of APP is lost in AD patients. Instead, APP mutations seem to act by a toxic gain-of-function mechanism. The APP mutations in the APP gene are clustered near the α-, β-, or γ-secretase cleavage sites, having a direct effect on APP processing namely, by increasing production of the potentially cytotoxic Aß fragment. For example, transgenic mice expressing the Swedish APP double mutation (KM670/671NL) produce numerous Aß deposits in the form of classical senile plaques (Bayer 2003) and exhibit an age-dependent increase in soluble Aß (1-40) and Aß (1-42) levels (Christi 2001, Gao 2002).

1.4.2 The beta amyloid peptide

As decribed above, the Aß peptide is generated by processing through ß- and γ-secretases. ß-secretase was recently identified (Yan 1999, Vassar 1999) as a transmembrane aspartyl protease known as BACE1 (b-site APP cleavage enzyme). BACE1 is expressed in all tissues with the highest level of expression in the brain. BACE1 shares 55% homology with a second enzyme called BACE2. Both enzymes contain the two characteristic D(T/S)G(T/S) motifs of aspartyl proteases, which form the catalytic site, and share significant sequence homology with other members of the pepsin family of aspartyl proteases. In contrast to all other aspartyl proteases of the pepsin family, BACE1 and BACE2 are type I transmembrane proteins with a large lumenal domain containing the active center, a single transmembrane domain, and a small cytoplasmic tail (Hong 2000). The γ-secretase is described in detail in 1.4.3.

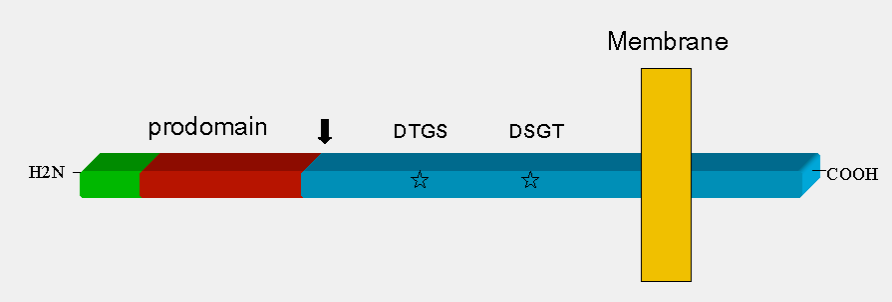

Fig. 1.4 Diagram of BACE. An aspartyl protease active site motifs (DTGS, DSGT) is localized in the lumenal/extracellular domain. During translocation and maturation through the secretory pathway the lumenal domain of BACE is N-glycosylated at four asparagine residues. As indicated by the arrow, during transport through the Golgi, the prodomain is endoproteolytically removed by a furin protease to generate the mature enzyme (Haas 2001).

To explain massive neuronal loss in the brain of AD patients, several models have been proposed. The dominant theory in the past decade has been the so-called "amyloid cascade" hypothesis, placing Aß as a principal culprit (Citron 1992; Yankner 1990; Selkoe 2002). The dysregulation in APP processing might occur early in the illness, leading to increased production of Aß. This in turn leads to a chain of downstream events including development of neurofibrillary pathology and cell death. The fact that a macromolecular form of Aß is toxic in *in vitro* studies by itself supports this hypothesis (Hardy 1992; Droet 1999). More importantly, as described before, rare dominant mutations causing FAD result in the accumulation of Aß.

The precise mechanisms whereby Aß leads to synaptic dysfunction and cell death in AD are not yet known, although there have been recent insights into this process. Possible mechanisms of Aβ toxicity include generation of free radicals (described in 1.6) and activation of apoptosis (see 1.5). An Aβ -induced perturbation of calcium homeostasis (Suo 1997, Gibson 2002, Eckert 2001b, Mattson 2003) and an alteration of the APP metabolism by cholesterol has also been proposed (Yanakisawa 2002, Mizuno 1999, Kirsch 2002).

Another question that still needs to be answered is: What kind of Aß peptide is responsible for neuronal cell loss? Aß42 is a hydrophobic peptide with an ominous tendency to assemble into long-lived oligomers and polymers. During the last decade, the extracellular, deposited, and fibriller Aß was culprit for the disease, since it is the main consistent of the senile plaques,

and synthetic Aß peptides can aggregate into an array of assembly forms, when added to cell culture. The problem thereby is that synthetic Aß peptides used *in vitro* have generally been applied at micromolar concentrations (in contrast to the low nanomolar levels of natural Aß found in the brain and cerebrospinal fluid). This aggregated forms may have biophysical and toxic properties unlike those found *in vivo*. Moreover, recent reports suggest that the toxicity of Aß lies not in the insoluble fibrils that accumulate but rather in the soluble oligomeric intermediates (Hardy 2002, Wilson 2003). Soluble Aß oligomers are also found in human AD cerebrospinal fluid, and the soluble Aß content of human brain correlated better with the severity of the disease than plaques. (Kuo 1996, Pitschke 1998, Lue 1999, McLean 1999).
Other experiments argue against the classical amyloid hypothesis which postulates that Aß deposition is the primary event triggering the pathological cascades leading to AD abnormalities, especially neuronal death. Studies of transgenic mice overproducing extracellular Aß have repeatedly observed the lack of neuronal loss in the presence of Aß deposition, suggesting that Aß deposition alone is not sufficient to cause neuronal death (Irizarry 1997). More recently, it was found that doubly transgenic mice expressing both the Swedish APP Mutation and the M146L-Presenilin 1 mutation show behavioral changes, but a lack of neuronal loss in the presence of Aß deposits (Holcomb 1999, Takeda 2004). Conversely, neuronal loss by a α-secretase-resistant mutant APP occurs without detectable extracellular Aß deposition (Moechars 1996).

The discrepancy between Aß deposition and neuronal loss as well as recent data pointed for intracellular Aß as a contributing factor or initiating event towards neuronal death and synaptic loss in early AD pathogenesis. Intracellular accumulation of Aß might impair cellular functions and may probably represent the primary event within the neurotoxic Aß cascade. (Wirths 2001). In cultured neuronal cells expressing APP, natural oligomers of Aß are formed soon after generation of the peptide within intracellular vesicels (Walsh 2002). A significant increase of intracellular Aß42-positive neurons in isolated cases of AD and in atypical AD cases not associated with dense core amyloid depositions was shown (Tabira 2002). In mutant PS1 transgenic mice the death of neurons is associated with intraneuronal Aß accumulation, but without extracellular Aß deposition (Chui 1999). Finally, it was found that the intracellular Aß1–42 mediates apoptosis of cortical neurons infected with adenovirus encoding APP (Kienlen-Campard 2002).

1.4.3 Presenilin

The concept that changes in APP processing are central to AD pathology won further support after the discovery that mutations in the presenilins interfere with the cleavage of APP, leading to an overproduction of Aß peptide. Mutations in the PS1 gene, located on chromosome 14, account for 18–50% of the early onset AD cases (Cruts 1998) and also cause the most aggressive forms of AD, in some cases with an onset below the age of 30. The majority of the over 120 known PS1 and 8 PS2 mutations are missense substitutions; only a couple of deletions and insertions have been reported to date (Tandon 2000).
PS1 and PS2 are integral proteins sharing 60% amino acid sequence homology and contain 6 to 8 transmembrane (TM) domains (Doan 1996). On the cytoplasmic side of the membrane they contain between TM domains 6 and 7 a large hydrophilic loop. The cytoplasmic loop includes the sites where proteolytic cleavages occur (see Fig.1.5). Full-length PS undergoes endoproteolysis to form stable N-terminal (NTF, 30 kDa) and C-terminal (CTF, 20 kDA) fragments (Thinakaran 1996). In 1999, it was hypothesized that PS1 could be the γ-secretase (Wolfe 1999a). This hypothesis was based on studies with inhibitors showing that γ-secretase has characteristics of an aspartyl protease (Shearman 2000; Wolfe 1999b). Identification of two conserved aspartate residues in TM domains 6 and 7 of PS critical for γ-secretase activity (D257 and D385 in PS1) provided the conceptual basis for the theory that PS is the aspartyl secretase. When either aspartate residues in PS1 (Wolfe 1999a) or PS2 (Kimberly 2000) are mutated, γ-secretase activity is blocked, and Aß levels are significantly reduced in cultured cells. The same was also observed in transgenic mice overexpressing D257A mutant PS1 (Xia 2001).

Despite these reports implicating presenilin as the γ -secretase, it has not been possible to increase substrate-cleaving activity by overexpressing PS1 and PS2 alone. This failure presumably relates to the observation that the endoproteolysis of presenilin heterodimers requires certain limiting cellular factors (Thinakaran 1996). Several factors apparently critical for γ-secretase activity have been identified to date. The presenilins are incorporated with nicastrin (Yu 2000) PEN-2 (Francis 2002) and Aph-1 (Goutte 2002) into a high molecular-weight protein complex of 200-250 kDa to form the γ-secretase. Presenilins appear to provide the active core of the protease while the other factors are needed for the assembly of the complex by stabilizing or proteolysing the Presenilin. Nicastrin is a glycosylated 130 kDa integral membrane protein that binds relatively well to both the NTF and the CTF of

Presenilin, while Aph-1 is a 30 kDa multimembrane spanning protein that is needed for the correct subcellular transport of Nicastrin to the cell surface. Pen-2 is a small, hairpin-like membrane protein of 12 kDa. All individual γ-secretase complex components might determine each other's expression. Taking away a single component disrupts the entire γ-secretase complex and consequently blocks intramembrane proteolysis (De Stropper 2003).

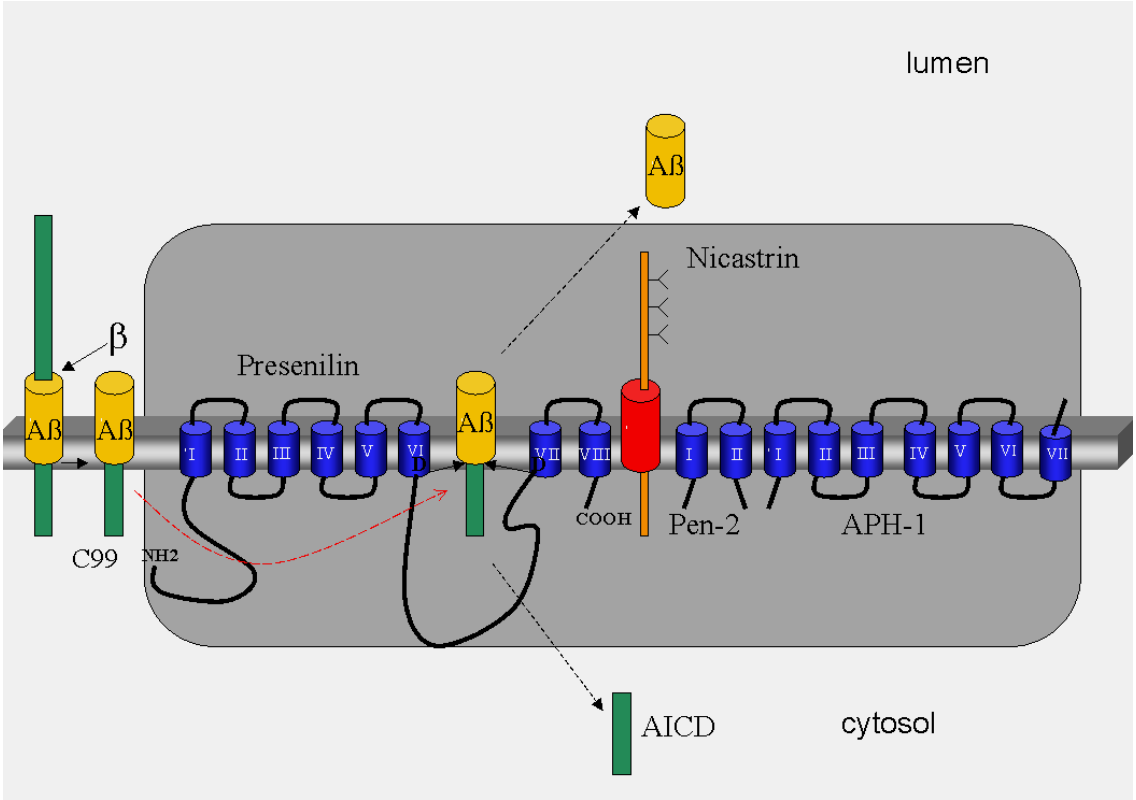

Fig. 1.5 Intramembrane proteolysis of APP by the γ-secretase complex according to Haass and Steiner (2002). After the cleavage of APP by ß-secretase, the generated C99 enters the active site of the γ-secretase residing in PS, which is itself part of a native 200–250-kDa complex containing nicastrin, PEN-2 and APH-1. The two crucial aspartates in TMDs 6 and 7 of PS mediate endoproteolysis, which results in the secretion of Aß and the liberation of AICD.

The deletion of the PS1 gene in mice resulted in an embryonic lethal phenotype (Shen 1997) because presenilins are also critical for processing of the Notch receptor, a signaling molecule crucial for cell-fate determination during embryogenesis (Wong 1997). After translation in the ER, Notch is processed by a Furin-like protease, resulting in a heterodimeric receptor that is shuttled to the cell surface (Fig. 1.6). Upon interaction with a cognate ligand, the ectodomain of Notch is cleaved by a metalloprotease apparently identical to tumor necrosis factor-a converting enzyme (TACE) (Mumm 2000). Interestingly, metalloproteases such as TACE and ADAM-10 are among the identified α-secretases that shed the APP ectodomain (Buxbaum

1998). The membrane associated C terminus is then cut by γ-secretase within the postulated transmembrane domain to release the Notch intracellular domain (NICD), which then translocates to the nucleus where it interacts with and activates the CSL family of transcription factors (Schroeter 1998). NICD formation is absolutely required for signaling from the Notch receptor. Interestingly in this context, the C-terminal fragment (AICD, see Fig. 1.3), composed of the last 57-59 amino acids of the C terminus of APP, has been demonstrated to cause transcriptional activation after translocalization into the nucleus, suggesting a role of AICD in gene regulation (Kinoshita 2002, Kim 2003).

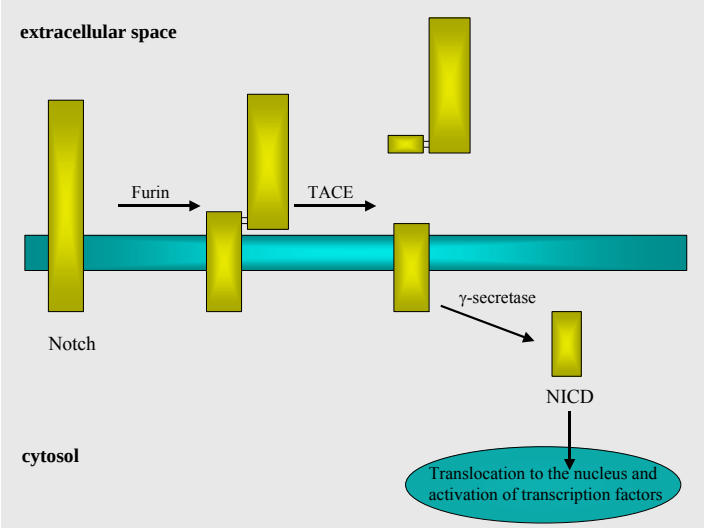

Figure 1.6. The processing of Notch occurs similar to APP. Notch signaling depends on three endoproteolytic cleavages. Notch maturates in the Golgi by furin-mediated cleavage. At the cell surface, Notch is cleaved by the metalloprotease TACE. Finally, PS-dependent cleavage liberates the NICD, which translocates to the nucleus, thereby regulating the transcription target genes by binding to transcription factors of the CSL family.

1.5 NEURONAL DEGENERATION IN ALZHEIMER'S DISEASE

1.5.1 Cell death mechanisms in central nervous system

Cell death occurs by necrosis or apoptosis. These two mechanisms have distinct histologic and biochemical signatures. In necrosis, the stimulus of death is itself often the direct cause for the death of the cell. In apoptosis, by contrast, the stimulus of death activates a cascade of events that orchestrate the destruction of the cell. Unlike necrosis, which is a pathologic

process, apoptosis is part of normal development (physiologic apoptosis). Its major function is to facilitate the survival of the organism by the controlled elimination of damaged or superfluous cells. Neuronal cells, for example, undergo extensive apoptotic death during development. However, apoptosis also occurs in a variety of diseases. Too less apoptosis is the causative factor for the origin of cancer. Too much apoptosis is a characteristic feature of many neurodegenerative disease.

1.5.1.1 Necrosis

Necrotic cell death in the central nervous system follows acute ischemia or traumatic injury to the brain or spinal cord (Linnik 1993). It occurs in areas that are most severely affected by abrupt biochemical collapse, which leads to the generation of free radicals and excitotoxins (e.g., glutamate, cytotoxic cytokines, and calcium). The histologic features of necrotic cell death are mitochondrial and nuclear swelling, dissolution of organelles, and condensation of chromatin around the nucleus. These events are followed by the rupture of nuclear and cytoplasmic membranes and the degradation of DNA by random enzymatic cuts in the molecule (Kanduc 2002, Kerr 1972).

1.5.1.2 Apoptosis

Apoptosis is an actively regulated process of cell death necessary for proper control of tissue growth. Apoptosis was originally described by Kerr et al. (1972) as an event characterized by plasma membrane blebbing, cell shrinkage, chromatin condensation, and degradation of DNA The word ‘apoptosis’ is derived from the Greek and literally means “the falling off of leaves from a tree, or the falling off of petals from a flower.” This definition came from early morphological observations of cells undergoing apoptosis. The surfaces of apoptotic cells have many blebs that look lile petal of flowers. In humans, ~100,000 cells die every second by apoptosis and are replaced by new cells of the same number. Apoptotic death is a tightly controlled process, and the cellular pathways that regulate apoptosis have been well conserved in multicellular organisms from simple to complex animals.

In the nervous system apoptotic cell death, also known as programmed cell death, can be a feature of both acute and chronic neurologic diseases (Yuan 2000, Martin 1999). After acute

insults, apoptosis occurs in areas that are not severely affected by the injury. For example, after ischemia, there is necrotic cell death in the core of the lesion, where hypoxia is most severe. Apoptosis occurs in the surrounding tissue. In chronic neurodegenerative diseases like Huntington's, Parkinson's or Alzheimer's Disease, apoptosis is the predominant form of cell death (La Ferla 1995, Thomas1995).

Apoptotic cell death is characterized by the activation of a biochemical cascade. Proteases termed as caspases (see. 1.5.2), destroy molecules that are required for cell survival and activate others that mediate a program of cell suicide. During the process, the cytoplasm condenses, mitochondria and ribosomes aggregate, the nucleus condenses, and chromatin aggregates. Finally, the cell is fragmented into "apoptotic bodies," and chromosomal DNA is enzymatically cleaved to 180-bp internucleosomal fragments. Other features of apoptosis are a reduction in the membrane potential of the mitochondria, intracellular acidification, generation of free radicals, and externalisation of phosphatidylserine residues (Kerr 1972, Thornberry 1998).

1.5.2 Caspases

Caspases (cysteine dependent, aspartate-specific proteases) are the major executioners of the apoptotic program. The first caspase initially discovered as a cytokine-processing enzyme was designated interleukin 1*b*converting enzyme (today also known as caspase 1). The discovery of this enzyme resulted from studies with the nematode *Caenorhabditis elegans.* The gene CED (cell death abnormal)-3 was found to be essential for apoptotic cell death in the developing worm (Yuan 1993). Database research revealed later the gene encoding for interleukin 1*b*converting enzyme as the mammalian homologue. 14 caspases have since been identified, 11 of them are present in humans (Mattson 2003). The numbering is in order of their discovery.

1.5.2.1 Caspase structure

Caspases are synthesized as zymogens or proenzymes (30–50 kDa) containing an N-terminal prodomain together with one large (p20 kDa) and one small (p10 kDa) subunit. Caspase activity requires proteolytic cleavage at aspartate residues separating these domains and the

association of two large and two small subunits into an active heterotetramer with two active sites (see Fig. 1.7).

Caspases play critical roles in the initiation or execution of apoptosis or, like caspase 1, in inflammation (Thornberry 1998). The prodomains of the caspase family fall into three groups. Prodomains of the initiator caspases tend to be long and contain protein interaction domains such as death effector domains (DED) or caspase recruitment domains (CARD). These domains, which share similar structural features, have a regulatory role by serving to couple cellular signaling pathways to caspase activation (Bratton 2000). Thus, the initiator caspases are usually the first to be activated upon integration of signals that commit the cell to die.

Activated initiator caspases then cleave and activate effector caspases containing a short prodomain. In turn, effector caspases cleave crucial cellular substrates executing the apoptotic cell death (see 1.5.3) The prodomains of the inflammatory caspases (see table 1.2) are often long and may contain regulatory domains, consistent with the need to control their proteolytic activity.

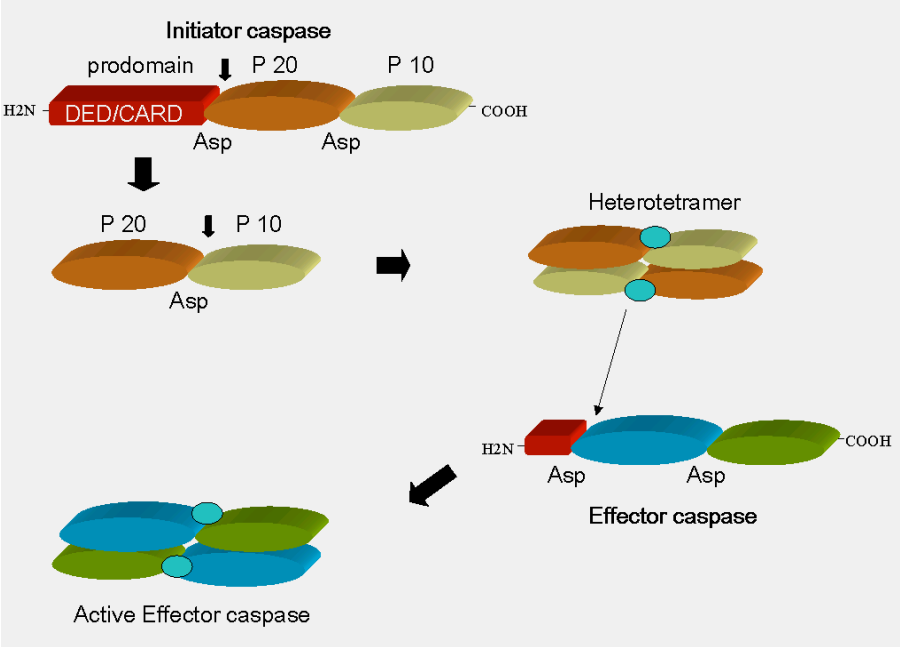

Figure 1.7. Mechanisms of Caspase Activation: Upstream initiator caspases are activated during the initiation of the cell-death cascade. They contain an prodomain, a large subunit, and a small subunit. Activated upstream caspase have autocatalytic activity and activate downstream effector caspases, which have a short prodomain as well as a large subunit and a short subunit.

Caspases are among the most specific proteases known. Each caspase recognizes a different tetrapeptide motif within a substrate and is characterized by almost absolute specificity for aspartic acid in the P1 position. Using a synthetic combinatorial tetrapeptide library, Thornberry et al. (1998) determined the preferred substrate cleavage site for several mammalian caspases (see table 1.2).

The catalytic mechanisms are similar for all caspases, since caspases hydrolyze peptide bonds on the carboxyl side of an aspartate residue (termed the P1 residue). During the cleavage of a substrate a tetrahedral intermediate is formed by His/Cys/Gly residues of the enzyme and the P1-Asp of the substrate. The specificity of caspases is due to structural feature. Since every caspase show considerable variation in both, length and amino acid composition of the active site, only the precise substrate is stabilized by hydrogen bonding in the substrate binding pocket. In addition to the presence of a consensus caspase site, the conformation of the target protein is also an important determinant of sensitivity to caspase cleavage. The structure of some caspases have been determined recently by X-ray diffraction. For example the structure of caspase 2 comprises two copies of the p20/p10 monomer. The core of the enzyme is formed by a 12-stranded ß-sheet, which is surrounded by a total of 14 α-helices (Schweizer 2003).

Name	Prodomain length (kDA)	Regulatory motif	Adapter molecule	Optimal Tetrapeptide
Initiator				
Caspase 2	51	CARD	RAIDD/ PACAP	DVAD/ DESD
Caspase 8	55	DED	FADD	IETD
Caspase 9	45	CARD	Apaf-1, Cyt C	LEHD
Caspase 10	55	DED	FADD	?
Caspase 12	50		?	?
Effector				
Caspase 3	32		Casp 8, 9,	DEVD
Caspase 6	34		Casp 3,7	VEID
Caspase 7	35		Casp 3, 8, 9,	DEVD
Cytokine processors				
Caspase 1,4,5,11,13,14	30 - 50	CARD	Inflammatosome	(W/Y,F)EHD

Table 1.2. Characteristics of caspases. Caspases can be divided into initiator caspases and effector caspases based on the presence of a large prodomain at their amino-terminal region. Initiator caspases containing a long prodomain, like caspase 2, caspase 8, caspase 9 and caspase 10 generally act in early stages of a proteolytic cascade, while effector caspases, like caspase 3, caspase 6 and caspase 7 act downstream, and are involved in the cleavage of specific cellular proteins. The third group consist of several caspases involved in the maturation of cytokines.

1.5.3 Apoptotic cell signaling

Apoptosis can be triggered by many different stimuli, including involvment of 'death receptors' (such as tumour necrosis factor and Fas ligand), growth factor insufficiencies, toxins, oxidative stress, and impaired calcium homeostasis. Thereby two pathways of caspase activation during apoptosis have been described.

1.5.3.1 Extrinsic apoptotic pathway:

In the extrinsic apoptotic pathway, death receptors, including CD95/Fas, DR4 and DR5 (death receptors 4 and 5; also called TRAIL-R1 and TRAIL-R2), as well as TNFR1 (tumor necrosis factor receptor 1), are activated by their cognate ligands, CD95 ligand, TRAIL (TNF-related apoptosis-inducing ligand) and TNF, respectively. Triggering of the receptors with their cognate ligands or agonistic antibodies results in receptor trimerization. Each of these receptors belongs to the TNF superfamily of receptors, and each activates caspase 8 by first recruiting the adapter proteins, FADD (Fas-associated death domain) and/or TRADD (TNF receptor-associated death domain). Death receptors possess both cysteine-rich extracellular domains and an intracellular cytoplasmic sequence known as the death domain (DD). The adapter protein FADD also contains a C-terminal DD, which enables it to bind to trimerized receptors through DD–DD interactions, as well as an N-terminal DED (death effector domain), which can associate with similar DEDs located in the prodomain of caspase 8. This complex of proteins is referred to as the DISC (death-inducing signalling complex) (Barnhardt 2003).

Following its activation within the complex, processed caspase 8 can in turn activate the effector caspase 3, which cleaves critical structural and regulatory proteins, resulting in apoptosis. Similarly, trimerized TNFR1 can recruit the adapter protein TRADD, which consequently recruits FADD and procaspase 8. TRADD however, also binds to the serine-threonine kinase RIP (receptor-interacting protein) and in turn, the adapter molecule RAIDD through a series DD–DD interactions. Interestingly, the N-terminus of RAIDD shares sequence similarity to the prodomains of caspase 2, and it recruits and activates procaspase 2 (Shearwin 2000).

Another initiator caspase involved in extrinsic apoptotic pathway is caspase 10. Like caspase 8, it also contains two DEDs in its N-terminal prodomain. However, it has been shown recently that caspase 10 cannot functionally replace caspase 8 in the DISC (Sprick 2002).

Thus, caspase-10 may serve as an inititator caspase only in some receptor mediated caspase cascades.

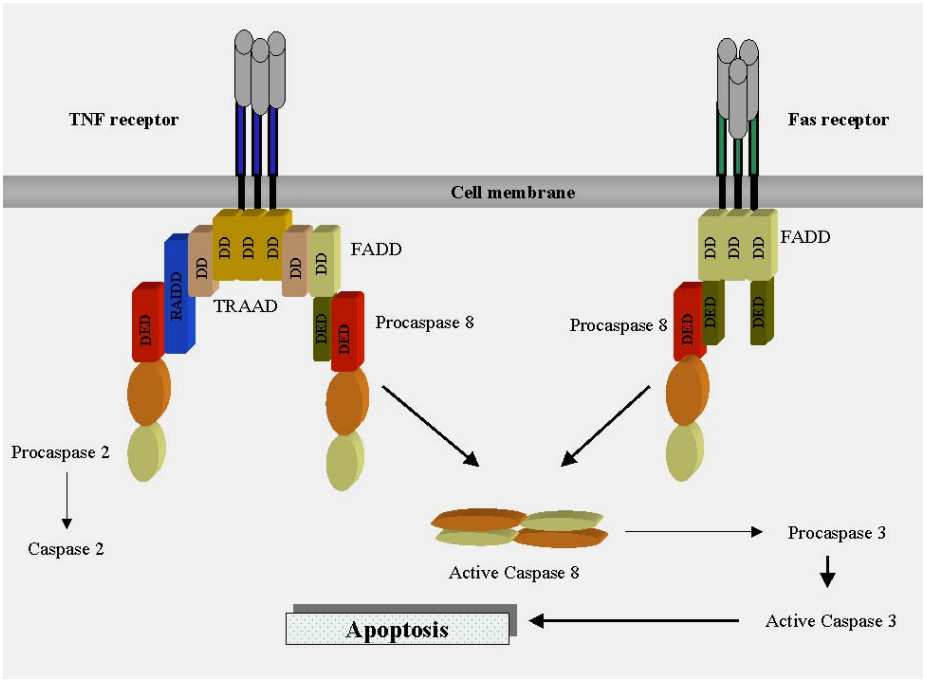

Fig.1 .8 Extrinsic apoptotic pathway

1.5.3.2 Intrinsic apoptotic pathway

In the second apoptotic pathway, diverse apoptotic signals converge at the mitochondrial level, inducing permeabilization of the outer mitochondrial membrane and the release of cytochrome C from the mitochondria into the cytosol. Once in the cytosol, cytochrome C binds to its cytosolic partner APAF-1 (apoptotic protease activating factor-1), the human homologue of *Caenorhabditis elegans* apoptotic protein CED-4, and induces the oligomerization of APAF-1-cytochrome *c* complex in a dATP/ATP-dependent manner (Green 1998). This heptameric complex, named "apoptosome," recruit the initiator caspase, procaspase 9, to the complex and induces procaspase 9 autoactivation (Cain 2003). The activated caspase 9 is released from the apoptosome and subsequently initiates a caspase cascade involving the effector caspases such as caspases 3 and 7 (Thornberry 1998). Two other proteins, Smac (also called Diablo) and Htr2A/Omi (Yang 2003), are also release from mitochondria and increase caspase activity by inactivating endogenous caspase inhibitors (IAP) (Verhagen 2000). Mitochondria also release apoptosis inducing factor (AIF) and endonuclease G, which appear to induce cell death independently of caspases by migrating to the nucleus and inducing chromatin condensation and large-scale DNA fragmentation.

Therefore, mitochondria are thought to be a central regulatory element in stress-induced apoptosis (Wang 2002).

The mitochondrial apoptotic pathway is regulated by proteins of the Bcl-2 superfamily. The Bcl-2 superfamily which consists of more than 20 members, are central regulators of apoptosis because they integrate diverse survival and death signals that are generated outside and inside the cell. The family is subdivided into two classes: anti-apoptotic members such as Bcl-2 and Bcl-xL (the Bcl-2-like survival factors) protect cells from apoptosis and generally contain four domains of sequence similarity, designated BH1 through BH4. Pro-apoptotic members are classified according to whether they contain one homology region (the "BH3-only" proteins, e.g., Bid, or Bim) or three (the "multidomain" or "BH1-3" proteins, including Bax, Bak, and Bok) (Cory 2002). It is still not entirely clear, how the 20 members of the bcl-2 superfamily regulate mitochondrial apoptotis. Each member of the BH3-only group might be activated in response to particular set of stimuli and through a characteristic mechanism. For example, Bid is cleaved by caspase 8 following death-receptor engagement, in turn Bim is involved in UV-induced apoptosis. Bax and Bak appear to permeabilize the outer mitochondrial membrane, allowing efflux of proapoptotic factors. Whereas Bax is predominantly a cytosolic monomer in healthy cells, during apoptosis it undergoes conformational changes, translocates to the outer mitochondrial membrane, and oligomerizes. In contrast, Bcl-2-like proteins prevent mitochondrial permeabilization such that none of the pro-apoptotic factors like cytochrome c or Smac are released to the cytosol (Kaufmann 2003).

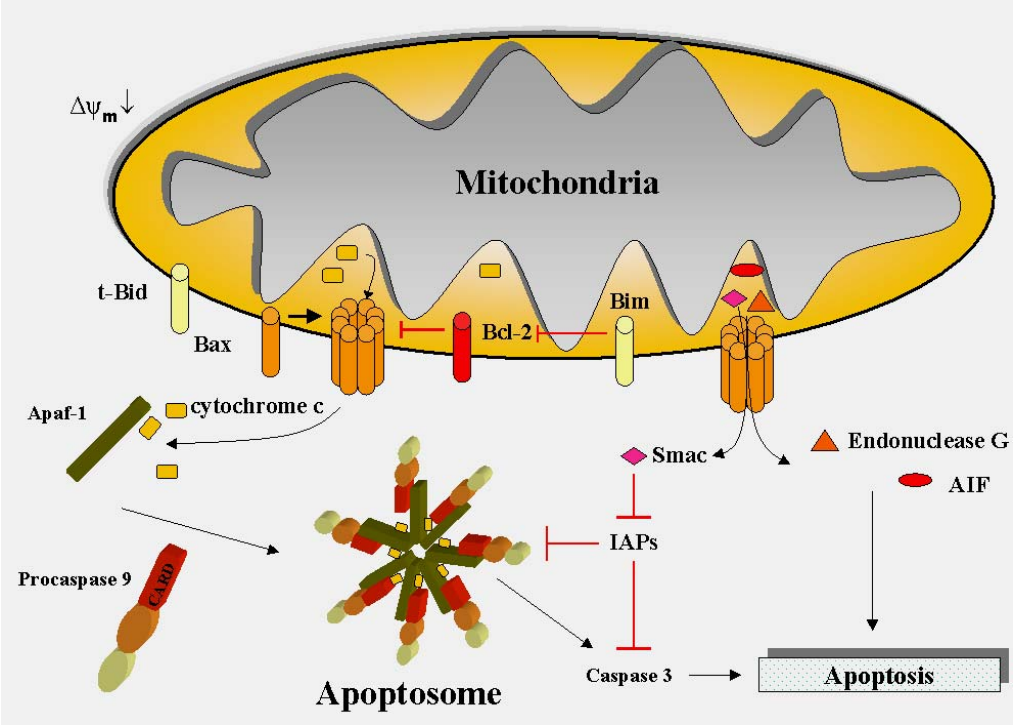

Fig.1 .9 Intrinsic apoptotic pathway

1.5.4 Caspase-Substrates

Caspases are extraordinarily selective enzymes. During apoptotic cell death, only a small fraction of the cellular proteome is cleaved by caspases. Comparative 2-D gel analysis revealed about 70 peptides as caspase "victims". These substrates, containing the sequence DX(E)X(V)D for caspase cleavage, are key structural components of the cytoskeleton and nucleus, or proteins involved in signaling pathways. The effects of these cleavage events are to disable homeostatic and repair processes, inactivate inhibitors of apoptosis, as well as mediate structural disassembly and morphological changes.
In order to mediate these events, caspases can modify the function of their target polypeptides in several ways (Earnhaw 1999):

- Caspases inactivate the normal biochemical function of their substrates. For example, one of the hallmark events of apoptotic cell death is genomic disassembly and oligonucleosomal fragmentation. Caspases disable normal DNA repair processes by inactivating at least two key proteins involved in the homeostatic maintenance of genomic integrity; poly(ADP-ribose) polymerase (PARP) and DNA-dependent serine/threonine kinase (DNA-PK). At the same time, an apoptosis-endonuclease (CAD, caspase activated desoxyribonuclease) is activated by caspase-mediated cleavage of its cognate inhibitor (ICAD).
- Caspases activate substrates by removal of regulatory domains (e.g. BID, PKCs,).
- Finally, the structural components of the cytoskeleton and nuclear scaffold require disassembly during apoptosis and caspases play a key proteolytic role in these steps as well (e.g. lamins, fodrin, gelsolin).
- Caspase cleavage normally occurs at a single, discrete site within the target polypeptide, although multiple cleavage has also been reported (ICAD).

1.5.5 Apoptosis and Alzheimer's disease

Apoptosis is of particular interest in the AD pathology. There is growing evidence that disturbances in the tight regulation of apoptosis may contribute to the progressive neuronal loss in AD brain. Convincing evidence by both, post-mortem examination (Su 1994, La Ferla 1995) and *in vitro* experiments (Forloni 1993, Loo 1993) indicate that neuronal cell death associated with Aß is apoptotic in nature. Aβ is believed to play a major role in promoting

neuronal degeneration and death by rendering neurons vulnerable to age-related increases in levels of oxidative stress and impairments in cellular energy metabolism (Mattson 2002, Gibson 2002). The underlying mechanisms of the Aß-induced apoptosis is still not understood. One possible mechanism for initiating apoptosis could be the generation of free radicals (Butterfield 1994) as discussed in 1.6. More evidences for the implication of apoptosis in AD come from experiments investigating AD mutations. Mutation in both, APP (Eckert 2001) and Presenilin 1 (Wolozin 1996, Terro 1992, Hashimoto 2002) sensitises cells for apoptosis.

As described in section 1.3 AD-causing mutations in Presenilins shift the normal processing pattern of APP by secretases toward an enhanced production of Aß peptide. In addition, Presenilin 1 is cleaved by caspase 3, generating a C-terminal fragment (PSCas); therefore an involvement of PS1 in the regulation of apoptotic proteolytic cascade has been proposed (Kim 1997). In addition to PS1, it has been shown that APP and PS2 are substrates of caspases *in vitro* and in cultured cells. (Gervais 1999, Kim 1997).

Despite these findings, it still remains unknown if PS1 have a direct regulatory function in apoptosis. Moreover, the function analysis of PS1 has been controversial. Due to pro-apoptotic reports of PS1, it has been hypothesized that caspase cleavage of presenilins and APP creates an apoptotic amplification cycle, accelerating cell death in the neurons of AD patients (Loetscher 1997 Kim 1997,). In contrast, persuasive evidences describing an antiapoptotic action of PS1 have also been shown (Roperch 1998; Imafuku 1999; Amson 2000). PSCas and ALG-3, a truncated form of PS2, may inhibit Fas induced apoptosis (Vezina 1998, Vito). PS1 assembles with the anti-apoptotic protein Bcl-2 into a macromolecular complex (Alberici 1999), and interact with a novel presenilin-associated protein (PSAP) (XU 2002). A negative regulation of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signaling pathway by presenilin 1 was proposed as the mechanism by which PS1 regulates apoptosis since overexpressed PS1 suppressed the SAPK/JNK activation in oxidative stress induced cell death (Kim 2001).

1.6 OXIDATIVE STRESS IN ALZHEIMER' S DISEASE

1.6.1 Generation of Reactive Oxygen Species

The brain is highly dependent on glucose for ATP generation necessary for many biochemical processes, and although the brain represents only 2% of the body mass, it accounts for 20% of the body's glucose consumption (Perry 2003). The key source for ATP is the mitochondrial electron transport chain. Electrons from nicotinamide adeninedinucleotide (NADH) and flavine adenine dinucleotide (FADH2), produced during the citric acid cycle, are transferred to molecular oxygen. At the same time, protons are translocate across the inner mitochondrial membrane. The consequence is the generation of a pH gradient across the inner membrane membrane that is utilized by ATP synthase for the production of ATP.

The mitochondrial electron transport chain consumes more than 90% of the oxygen taken up by the cell. From this about 1–5% are converted into superoxide $O2^-$ during normal physiological state (Aliev 2003). $O2^-$ belongs to a group of radical termed as reactive oxygen species (ROS), recognized as potentially toxic by-products of cellular metabolism. $O2^-$ is converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 in turn is reduced to water by glutathione peroxidase (GPx). If this does not occur, in the presence of divalent cations such as iron or copper H_2O_2 can undergo Fenton reaction and produce hydroxyl radicals (OH^-).

The free radical hypothesis of aging, proposed by Harman in 1992, postulated that the age-related accumulation of ROS results in damage of major components of cells: nucleus, mitochondrial DNA, membranes, and cytoplasmic proteins. The imbalance between the generation and elimination of ROS may be involved in the pathogenesis of most of the neurodegenerative disorders, including AD. Neurons appear to be particularly vulnerable to attack by free radicals, because their glutathione content is low (Cooper 1997) and brain metabolism requires substantial quantities of oxygen. Since age is the major risk factor for AD, and one of the earliest pathological events in AD is oxidative damage in brains of affected individuals (Smith 2000, Bayer 2003), free radicals may play a key role in the development of the disease.

Recent reports show an H_2O_2 induced increase in the concentrations of both intracellular (Misonou 2000) and bio-secreted Aß (Olivieri 2001). Simultaneously, Aβ is believed to promote neuronal degeneration and death by rendering neurons vulnerable to increases in levels of oxidative stress and impairments in cellular energy metabolism (Mattson 2002, Gibson 2002).

How genetic factors of AD are related to oxidative is unknown. It is assumable, that some AD related genetic factors (see. 1.3) lead to enhanced oxidative stress. In fact, increased oxidative stress levels have been found in the temporal inferior cortex from Swedish FAD patients (Bogdanovic 2001) and brains of mice transgenic for human presenilin 1 showed reduced antioxidative enzyme activity (Leutner 2001).

1.6.1 Stress-activated Protein Kinase and AD

Alterations in gene expression and enzyme activity, induced by extracellular stimuli/stress, are mediated through the interplay of multiple signaling pathways. Among these are the highly conserved mitogen-activated protein kinase (MAPK) pathways, which are the central mediators that propagate signals from the membrane to the nucleus. MAPKs comprise a group of serine/threonine kinases that are activated through multiple protein kinases by dual phosphorylation at conserved threonine and tyrosine residues All eukaryotic cells possess multiple MAPK pathways, each of which is preferentially activated by distinct sets of stimuli, thereby allowing the cells to respond coordinately to multiple divergent inputs. MAPK pathways play important roles in cellular processes from gene expression, cell cycle control, proliferation, differentiation, inflammation and cell death (Grewall 1999).

In neuronal cells, potentially deleterious stimuli, such as deprivation of trophic factors, UV irradiation, free radicals, hypoxia, ischemia, heat shock, and cytokines, provoke an intracellular stress response that either leads to apoptosis or defensive-protective adaptations. Stress-activated protein kinase (SAPK, also know as c-Jun N-terminal kinase; JNK) pathways and its downstream effectors are the major cellular factors involved in this response, which can lead to neurodegeneration or neuroprotection depending on the cellular and environmental conditions, as well as the influence of other signalling pathways (Herdegen 1997).

Since JNK activation plays an important role in oxidative stress signalling as reported recently (Zhu 2001, Aoki 2002), there is increasing interest in understanding the role that JNK may play in the pathogenesis of AD. Oxidative stress may cause the activation of c-Jun N-terminal kinase in degenerating neurons in AD (Suzaki 2002). Other models reported the involvement of JNK pathway by the induction of Fas ligand in Aß-induced neuronal apoptosis (Morishima 2001) and in TNF-induced induced cell death (Dietrich 2003).

JNK is also implicated in the modulation of APP metabolism since JNK induces phosphorylation of Thr668 within the carboxy-terminus of APP, a phosphorylation which is believed to regulate the function and metabolism of APP (Standen 2001). Furthermore, the findings that JIP1b, the JNK scaffolding protein, strongly binds to the cytoplasmic domain of APP (Taru 2002a, Scheinfeld 2002) at the YENPTY-motif, prompt the suggestion that JIP1b facilitates JNK accession to, and phosphorylation of, APP. This is supported by the finding that JNK associates with AßPP via JIP1b (Matsuda 2001) and that the overexpression of JIP-1b enhances the JNK dependent threonine phosphorylation of APP (Taru 2002b).

Since APP is able to trigger intracellular signalling pathways, it is assumable that APP may regulate the JNK pathway via JIP-1b as a receptor. JIP-1b my act as a scaffold protein between APP and JNK (Inomata 2003) and link APP and kinesin light chain-1 (Matsuda 20003). JIP-1b interaction with APP stabilizes immature APP and inhibits Aß secretion *in vitro* (Taru 2002b). The kinesin–JIP-1b–APP complex may mediate fast axonal transport of vesicles containing APP, presenilin, and BACE (Kamal 2001). JIP-1b could serve as cargo for the microtubule motor kinesin to mediate the transportation of several transmembrane proteins in which APP is supposed to be a cargo receptor, probably the physiological role of APP. A disruption of the kinesin-JIP-1b-APP complex assembly could be the origin for the activation of JNK, leading to phosphorylation of APP and proteolytic cleavage by ß- and γ-secretase.

Interestingly, JNK is also associated to phosphorylation of Tau. It was shown that JNK can phosphorylate Tau in neuronal cells (Bue´e-Scherrer 2002). Additionally, JNK activation correlates with increased phosphorylated Tau in the brains of mice (Planel 2001). Thus, JNK activation may link both neuropathological hallmarks of AD; Aß production and the

formation of intracellular neurofibrillary tangles containing abnormally phosphorylated tau protein aggregated into filaments.

1.7 AIM OF THESIS

Massive and progressive neuronal loss is the central abnormality occurring in brains suffering from Alzheimer's disease (AD), in addition to senile plaques and neurofibrillary tangles. The elucidation of the mechanisms involved in neuronal death in AD is therefore of particular importance in order to develop therapeutic targets. Strong evidence indicates that responsible for this massive neurodegeneration, affecting probably 20–30 million people worldwide, is a small peptide called amyloid beta (Aß). It arises by proteolysis from the amyloid precursor protein (APP) through an initial β-secretase cleavage followed by an intramembraneous cut of the γ-secretase complex. Aß is generated first intracellulary and then exposed to extracellular deposits accumulated principally in a filamentous form in neuritic plaques. The neurotoxicity of Aß has been associated with different events such as oxidative stress, altered calcium regulation, mitochondrial defects, excitatory toxicity, and deficiency of survival factors.

Although the cause of sporadic AD is yet unknown, certain rare familial AD (FAD) cases are caused by mutations in several genes. Missense mutations located within the APP-gene and immediately flanking the beta amyloid (Aß) sequence were the first familial AD-gene to be identified. The two other genes implicated in the early onset FAD are presenilins (PS) 1 and 2, causing the most aggressive form of AD known, with onset commonly occurring in the 40s and 50s and very rarely as early as 30s. Little is understood of how these mutant genes lead to these early onset forms of AD. Interestingly, FAD and sporadic AD have common clinical and neuropathological features. Therefore, using transgenic models expressing FAD-mutations are promising approaches to elucidate the underlying mechanisms of neuronal death in AD. On the basis of many studies in cell cultures, transgenic animal models, and patients, we know that FAD mutations alter APP processing with respect to an enhanced Aß production.

Based on results which indicate that Aß promotes neuronal degeneration and death by rendering neurons vulnerable to increase in levels of oxidative stress, the main aim of this thesis was to elucidate the neurotoxic biochemical pathways induced by Aß, investigating the effect of the APP Swedish double mutation (APPsw, KM670/671NL) on oxidative stress-induced cell death mechanisms. This mutation results from a three- to sixfold increased Aß production compared to wild-type APP (APPwt). As cell models, the neuronal PC12 (rat pheochromocytoma) and the HEK (human embryonic kidney 293) cell lines were used, which

has been transfected with human wiltyp APP or the human APP containing the Swedish double mutation. The PC12 cell model offers two important advantages. First, compared to experiments using high concentrations of Aß at micromolar levels applied extracellularly to cells, PC12 APPsw cells secret low Aß within picomolar range reflecting physiological production in Ad brain. Thus, this cell model represents a very suitable approach to elucidate the AD-specific cell death pathways mimicking pathological conditions. Second, these two cell lines (APPwt and APPsw) with different production levels of Aß may additionally allow to study dose-dependent effects of Aß. In addition, APP-transfected HEK cells exhibit a 30-fold increased Aß production as APP-transfected PC12 cell, representing a model of high chronic Aß stress, allowing to study further dose-dependent effects of Aß.

Another aim of the present study is based on previous findings showing an anti-apoptotic role of Presenilin 1. Presenilin 1 is an aspartyl protease, involved in the γ-secretase mediated proteolysis of Aß. Recent studies have suggested an additional role for presenilin proteins in apoptotic cell death observed in AD. Since Presenilin 1 is proteolytically cleaved by caspase 3, it has been proposed that the resulting C-terminal fragment of PS1 (PSCas) could play a role in signal transduction during apoptosis. Moreover, it was shown that mutant presenilins causing early-onset FAD may render cells vulnerable to apoptosis. The mechanism by which PS1 regulates apoptotic cell death is yet not understood. Therefore, it should be investigated if Presenilin 1 is involved in the proteolytic apoptotic cascade and whether the cleavage of PS1 by caspase 3 has an regulatory function. As cell models, PC12 and Jurkat T-cell lymphoma cells were transfected with human full lengths Presenilin 1, the caspase 3 cleaved C-terminal fragment of PS1 (PSCas) or full lengths Presenilin 1 with a mutated caspase 3 recognition site (D345A/ PSMut), which inhibits cleavage of PS1 by caspase 3. Finally, the effects of these transfections with regard to apoptotic vulnerability and apoptotic mechanisms were analyzed.

1.8 ZIELE DER ARBEIT

Massiver und fortschreitender neuronaler Zelltod gehört zu den zentralen Charakteristika der Alzheimer Demenz (AD), ebenso wie Senile Plaques und Neurofibrillenbündel. Die Aufklärung der Mechanismen die zum neuronalen Zelltod führen sind daher von entscheidender Bedeutung bei der Suche nach neuen therapeutischen Ansätzen. Verantwortlich für den neuronalen Zelltod ist das Amyloid-Beta Peptid (Aß), das ein proteolytisches Spaltprodukt des Amyloid Precursor Proteins (APP) ist. Die Spaltung von Aß erfolgt hierbei initial durch die ß-Sekretase und anschließend intermembranär durch den γ-Sekretase Komplex. Aß wird intrazellulär generiert und extrazellulär in aggregierten und unlöslichen Plaques angereichert. Die Mechanismen der Aß-Toxizität sind noch nicht genau bekannt; assoziiert werden oxidativer Stress, veränderte Kalziumhomöostase, mitochondriale Defekte und der Verlust an Überlebensfaktoren.

Obwohl die Ursachen der sporadischen AD nicht bekannt sind zeigen genetische Analysen, dass Mutationen im APP-Gen auf Chromosom 21 die familiäre Form der Alzheimer Erkrankung (FAD) verursachen. Daneben wurden zwei andere Loci auf Chromosom 14 (Presenilin 1) und Chromosom 1 (Presenilin 2) mit der early-onset FAD beschrieben. Mutationen im Presenilin 1 oder Presenilin 2 Gen führen zu einem frühen Beginn der Erkrankung, zum Teil schon vor dem 40. und 50. Lebensjahr. Wie diese mutierten Gene zur early-onset FAD führen, ist fast völlig unbekannt. Interessanterweise weisen FAD- und sporadische AD-Fälle die gleichen klinischen und neuropathologischen Eigenschaften auf. Daher bieten transgene FAD-Modelle vielversprechende Ansätze um die zugrundeliegenden Mechanismen des neuronalen Zelltods bei der AD aufzuklären. Aufgrund von Studien mit Zellkulturenmodellen, transgenen Tiermodellen und Patienten, wissen wir, dass FAD Mutationen die APP Prozessierung beeinflussen und eine erhöhte Aß Produktion bewirken.

Basierend auf Ergebnissen, die darauf hinweisen, dass bei AD eine veränderte Vulnerabilität gegenüber den oxidativen Stress induzierten Zelltod vorliegt, sollten im Rahmen dieser Arbeit die vom Aß eingeleiteten neurotoxischen biochemischem Signaltransduktionswege aufgeklärt werden. Hierfür wurde der Effekt der schwedische APP Doppelmutation (APPsw, KM670/671NL) auf den durch oxidativen Stress induzierten Zelltod untersucht. Diese Mutation die im Rahmen der FAD vorkommt, verursacht eine drei- bis sechsfach erhöhte Aß Produktion im Vergleich zu humanen wild Typ APP (APPwt). Als Zellmodell dienten PC12- und HEK-Zellen, die mit Vektoren transfiziert wurden, die einerseits APPwt, und anderseits

APPsw enthalten. Das PC12 Zellmodell bietet hierbei zwei wichtige Vorteile. 1. Verglichen mit Experimenten bei denen Aß extrazellulär im mikromolaren Bereich appliziert wird, sekretieren PC12 APPsw Zellen, entsprechend der physiologischen Produktion im Gehirn von AD Patienten, Aß im pikomolaren Bereich. 2. Beide Zelllinien (APPwt und APPsw) haben eine unterschiedliche Aß Produktion, was die Untersuchung von dosisabhängigen Effekten ermöglicht. APP-transfizierte HEK Zellen weisen eine 30fach erhöhte Aß Produktion im Vergleich zu den APP-transfizierte PC12 Zellen auf, und stellen ein Modell für chronisch erhöhte Aß-Spiegel dar. Dies erlaubt weitere dosisabhängige Untersuchungen von Aß.

Ein weiteres Ziel dieser Arbeit basiert auf vorhergehenden Hinweisen, die auf eine anti-apoptotische Rolle von Presenilin1 (PS1) hindeuten. PS1 ist eine Aspartyl-Protease, involviert in der Proteolyse von Aß durch den γ-Sekretase Komplex. Neue Daten lassen auf eine zusätzliche Rolle von PS1 im Rahmen des apoptotischen Zelltods bei der AD schließen. Da PS1 proteolytisch durch die Caspase 3 gespalten wird, wurde postuliert, dass das resultierende C-terminale Fragment von PS1 (PSCas) eine Rolle während der apoptotischen Signaltransduktion haben könnte. Zusätzlich wurde gezeigt, dass FAD mutiertes PS1 zu einer erhöhten Vulnerabilität von Zellen führt. Wie PS1 den apoptotischen Zelltod reguliert, ist unbekannt. Daher wurde im Rahmen dieser Arbeit untersucht, ob PS1 in der proteolytischen apoptotischen Kaskade involviert ist, und ob die Spaltung von PS1 durch Caspase 3 eine regulatorische Funktion besitzt. Als Zellmodelle wurden PC12 und Jurkat T-cell lymphoma Zellen verwendet ,die transfiziert wurden mit:

1. humanem PS1
2. Caspase 3 gespaltenem C-terminalem Fragment von PS1 (PSCas)
3. mit Caspase-3 mutierter Erkennungssequenz (D345A/ PSMut), was die Spaltung von PS1 durch Caspase 3 verhindert.

Schließlich wurden die Effekte der Transfektionen in Hinblick auf die Zellvulnerabilität und Apoptosemechanismen untersucht.

2. Material and Methods

The material listed below include the used laboratory equipment and utensils. The used buffers, enzymes, chemicals or antibodies are listed under the appropriate methods step.

2.1 APPARATUS:

Agitator, type REAX 2000, Heidolph, Merck Eurolab GmbH, Frankfurt
Biomax™ TranScreen-LE, Eastman Kodak, Rochester, New York, USA
Branson Sonifier, Cell Disruptor B15, Branson Ultrasonics Corp., Danbury
Centrifuge, model GS-6R, Beckman, München
Centrifuge, model J2-with JA-20 und JA-20.1 rotor, Beckman, München
CO_2-incubators Heraeus, Typ BB 6220, Heraeus Instruments GmbH, Hanau
Combitips 0.5 ml, 2.5 ml, 10 ml, Eppendorf-Netheler-Hinz-GmbH, Hamburg
Coverslips for haemacytometer (Neubauer chamber), Superior, Merck Eurolab GmbH, Frankfurt
Cryo-vial, model Cryo-S, 2 ml, Greiner Labortechnik, Frickenhausen
Culture dishes and flasks, Corning® 25 cm^2, Polystyren, Dunn Labortechnik, Asbach
Culture vessels, IWAKI Dunn Labortechnik / Greiner Labortechnik Frickenhausen
Developer, Replenisher, GBX reagents, Eastman Kodak, Rochester, New York, USA
Electrophoresis, transfer-chamber Mini-Trans-Blot, BioRad, München
FACSCalibur™ (E1034), Becton Dickinson, Heidelberg
Fluorescence microscope, Eclipse E 800, Nikon, Japan
Fluorescence spectrometer, Victor Multilabel counter, Perkin Elmer, Jügesheim
Horizontal slab gel electrophoresis apparatus 10 x 15 cm, PeqLab Biotechnologies GmbH, Erlangen
Incubator for bacteria, Kelvitron kl. Heraeus Instruments GmbH, Hanau
Inverse microscope, model TMS, type I04, Nikon, Japan
Laminar flow hood, Heraeus InstrumentsGmbH, Hanau
Latex examination gloves, Ansell GmbH, München
Liquid scintilattion counter, Wallac 1409 Berthold, Turku, Finland
Magnetic agitator, type Poly 15, H + P Labortechnik, Merck Eurolab GmbH, Frankfurt
Microtiter plates IWAKI, 96 well with flat bottom, Dunn Labortechnik GmbH, Asbach
Microtiter plate, U-shaped botttom, sterile, Greiner Labortechnik, Frickenhausen
Microcentrifuge, type GS-6R Centrifuge, Beckman, Krefeld
Multipette® plus 4981, Eppendorf, Merck Eurolab GmbH, Frankfurt
Neubauer chamber Superior, Merck Eurolab GmbH, Frankfurt
Parafilm® M, Merck Eurolab GmbH, D-60487 Frankfurt
pH-meter, type CG 825, Schott, D-65719 Hofheim
Photometer for microplate model Digiscan, Firma Asys Hightech, Eugendorf
Pipetus®-akku, Hirschmann, Merck Eurolab GmbH, Frankfurt
Plastibrand® Tip-Rack, 5 – 300 µl, Brand, Merck Eurolab GmbH, Frankfurt
Pipettes model Pipetman 10, 20, 200, 1000 und 5000 µl, Abimed, Langenfeld
Pipettes sterile packed 5, 10 and 25 ml, Greiner Labortechnik, Frickenhausen
PP-tubes 15 ml and 50 ml, Cellstar®, Greiner Labortechnik, Frickenhausen
PVDF-ImmobilonTM-P-transfermembran, Millipore, Eschborn
Thermocycler, GeneAMP PCR-9700, Perkin Elmer Bio Systems, USA
Transferpette®-8, 10 - 100 µl, Brand, Merck Eurolab GmbH, Frankfurt
Transferpette®-8, 30 - 300 µl, Brand, Merck Eurolab GmbH, Frankfurt
UV/VIS-photometer, type U-2000 Spectrophotometer, Hitachi, Düsseldorf
Vertical slab gel electrophoresis apparatus, Bio-Rad, München
Water bath, type Thermomix 1441, B.Braun-Melsungen, Düsseldorf
Water bath, type 1003, GFL, Merck Eurolab GmbH, Frankfurt
Weighing scale machine, model Ab204 and AT261, Mettler, Gießen

2.2 CELLBIOLOGICAL METHODS

2.2.1 Culture of cell lines

All here described experiments with cell lines were conducted under sterile conditions in a laminar flow box. Cells were kept at 37°C, 94% humidity and 5% CO_2 in sterile culture vessels. All buffers and solutions were made from commercially available chemicals of analytical grade using purified (Millipore MilliQ-Plus) or double distilled water and were autoclaved or sterile filtered prior to use.
Media were heated to 37 °C prior to use. To separate adherend Cells from culture dishes a mix of EDTA/Trypsin was used. Cells were routinely passaged and washed with phosphate buffered saline (PBS). In regular time intervals cells were checked for Mycoplasma contamination by perfoming a Mycoplasma PCR .

PBS	Invitrogen
EDTA/Trypsin (0,25% EDTA/1 mM Trypsin)	Invitrogen
Mycoplasma Plus™PCR Primer Kit	Stratagene

2.2.2 Used cell lines

The following cell lines were used:

PC12 cells (rat pheochromocytoma) ref. (Greene et al. 1976):

a) untransfected PC12 cells

The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 5% heat-inactivated horse serum, 50 U/ml penicillin and 50 µg/ml streptomycin.

D-MEM complete medium	Invitrogen
10% FCS (fetal calf serum)	Invitrogen
-5 % HS (Horse Serum)	Invitrogen
-50 µg/ml (1%) Penicillin	Invitrogen
-50 µg/ml (1%) Streptomycin	Invitrogen

b) PC12 cells transfected with pCMV-vector containing:

- human wildtyp APP (APPwt)
- human APP containing the swedish double mutation KM670/671NL (APPsw)

- empty pCMV- vector (control).

PC12 cells were transfected with DNA constructs harbouring human mutant (APPsw, KM670/671NL) or wild-type APP (APPwt) gene, inserted downstream of a CMV promotor, using FUGENE technique (Roche Diagnostics). The stably transfected clones (APPwt M5, N10, U7 and APPsw Q8, Q9) were selected based on their similar expressoon of APP695 and the fivefold increased secretion of Aß(1-40) in the APPsw clones.
The pCMV695-vector contains a neomycin-resistance cassette, allowing to select stably transfected cells to be selected using G418.

c) PC12 cells transfected with a pcDNA3-vektor encoding:

- human presenilin 1 (PS1)
- human presenilin 1 with a mutated caspase 3 cleavage site D-345-A, (PS1mut)
- a C-terminal human presenilin 1 fragment processed by caspase 3 cleavage (PS1Casp)
- empty pcDNA3-vektor (A3)

These cells were stably transfected by Lipofectamine 2000 reagent (The procedure is described in 2.2.5). Stably transfected cells were selected using G418 on the basis that pcDNA3-vector contains a neomycin- resistance cassette. The effective transfection of the different contructs were verified by RT-PCR.

Transfected PC 12 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 5% heat-inactivated horse serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 400 µg/ml G418.

D-MEM complete medium	Invitrogen
10% FCS (Fetal Calf Serum)	Invitrogen
-5 % HS (Horse Serum)	Invitrogen
-50 µg/ml (1%) Penicillin	Invitrogen
-50 µg/ml (1%) Streptomycin	Invitrogen
400 ug/ml G418	PAA Laboratories

HEK cells (human embryonic kidney 293) ref. (Shigematzu et al. 1971)

a) untransfected HEK cells

The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin.

D-MEM complete medium	Invitrogen
-5 % HS (Horse Serum)	Invitrogen
-50 µg/ml (1%) Penicillin	Invitrogen
-50 µg/ml (1%) Streptomycin	Invitrogen

b) transfected HEK cells with pCMV-vector containing:

- human wildtyp APP (APPwt)
- human APP containing the swedish double mutation KM670/671NL (APPsw)

Transfected HEK cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 400 µg/ml G418.

D-MEM complete medium	Invitrogen
10% FCS (fetal calf serum)	Invitrogen
-50 µg/ml (1%) Penicillin	Invitrogen
-50 µg/ml (1%) Streptomycin	Invitrogen
400 ug/ml G418	PAA Laboratories

Jurkat T-cell lymphoma cells

The cell suspension of Jurkat T-cell lymphoma cell line was maintained in RPMI culture medium containing 10% FCS and Penicillin/ Steptomycin.

RPMI-culture medium:	
-10% FCS (2 hours heat inactivated at 56°C)	Invitrogen
- Penicillin/ Streptomycin 1%	Invitrogen
→ in RPMI	Invitrogen

Using Lipofectamine 2000 reagent (see 2.2.5), transient transfected Jurkat cells were generated by transfection with pcDNA3-vector encoding for:

- human presenilin 1 (PS1)
- human presenilin 1 with a mutated caspase 3 cleavage site D-345-A, (PS1mut)
- a C-terminal human presenilin 1 fragment processed by caspase 3 cleavage (PS1Casp)
- empty pcDNA3-vektor (A3)

2.2.3 Cell counting and viability test

To resolve the cell number, cells were resuspended with care and mixed with trypan dye solution (10:1). An aliquot of the stained cell suspension was subjected to a Neubauer chamber. Trypan dye penetrate into death cells due to a disrupted cell membrane, while vital cells are undyed. The arithmetical mean from different counting sections is determined and the cell number calculated in respect to the chamber factor and the dilution.

Trypan blue dye	Invitrogen

2.2.4 Cryoconservation of cells

Cells were washed with PBS, collected by centrifugation and all supernatant removed. Cells were resuspended in freezing medium, containing DMSO, in order to prevent harm by ice crystal formation during storage under freezing conditions. Cells were subjected quickly on ice for several minutes and than stored overnight in a freezer (-20°C). The following day, the cells were subjected in an -80°C freezer overnight and than stored for in liquid nitrogen.
To thaw cells, cryotubes containing the desired cells were rapidly subjected from the freezer or the nitrogen tank to a prewarmed (37°C) water bath for one or two minutes, until the solution is thawed. The cells were immediately diluted in a large amount of prewarmed complete culture medium, and washed several times by gentle centrifugation. Finally the cells were seeded in a culture vessel.

Freezing medium:	
-50% FCS	Invitrogen
-10% DMSO (dimethylsulfoxide)	Sigma
→ in medium	

2.2.5 Transfection of cells

The transfection of PC 12 and Jurkat cells were performed using the Lipofectamine™ 2000 reagent. The principle of the transfection is the uptake of an endocytotic DNA/cationic lipid complex by the cell. The day before transfection, PC 12 cells were counted and plated in a 24 well plate at 3 X 10^5 cells per well so that they were 90% confluent on the day of transfection.

Cells were plated in 0.5 ml of their normal growth medium containing serum without antibiotics.
For each well of cells, 0.8 µg of DNA were deleted in 50 µl of OPTI-MEM Reduced Serum Medium without serum. At the same time for each well of cells, 2 µl of Lipofectamin 2000 were diluted in 50 µl OPTI-MEM I Medium and incubate for 5 min at room temperature. The Lipofectamin reagent and the diluted DNA were mixed and incubate for 20 min, to allow the formation of DNA-Lipofectamin complex. Finally the DNA-Lipofectamin complexes (100 µl) were added directly to each well and mix gently.

The cells were incubated at 37°C in a CO_2 incubator for 24 hr washed with PBS and splitted in a high ratio to allow the identification of individual clones Individual clones were the incubate for 7 days in D-MEM containing 10 % FCS, 5 % HS, 1% Penicilin, 1 % Streptomycin and a high concentration of G418 (800 µg/ml), to allow the selection of positive transfected clones stably expressing the inserted gene.

Lipofectamine 2000	Invitrogen
Optimem-medium	Invitrogen

Jurkat cells were transfected transiently. The principle of the transfection is the same as for the PC12 cells, with the difference that after the addition of the DNA-Lipofectamine complexes, cells were incubated at 37°C in a CO_2 incubator for 4 hr and the OPTI-MEM replaced with the growth medium. After 24 cells were assayed for experiments.

2.3 GENETIC ENGINEERING

All the molecular biological work steps were performed under sterile conditions in order to avoid digestion of nucleic acids by DNases and RNases as well as the contamination by strange DNA. Hence, only sterile disposable plasticware, glass, pipettes and other material were used. The used solutions and buffers were autoclaved before use. Furthermore, during all working steps one-way gloves were worn.

2.3.1 Plasmids

The following plasmids were used:

a) pCMV, b) APPwt-pCMV, c) APPsw-pCMV

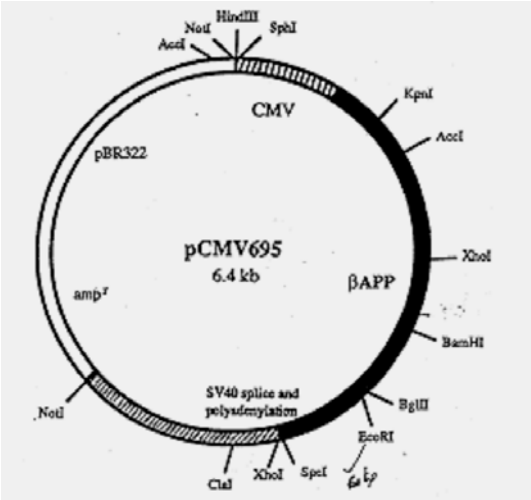

Figure 2.1. APP-pCMV695 vector. The human APP is under the control of the CMV promotor. The vector also contains an ampicillin resistance for selection.

d) pcDNA3 e) pcDNA3-PS1wt, f) pcDNA3-PS1Mut (D345A), g) pcDNA3-PSCas

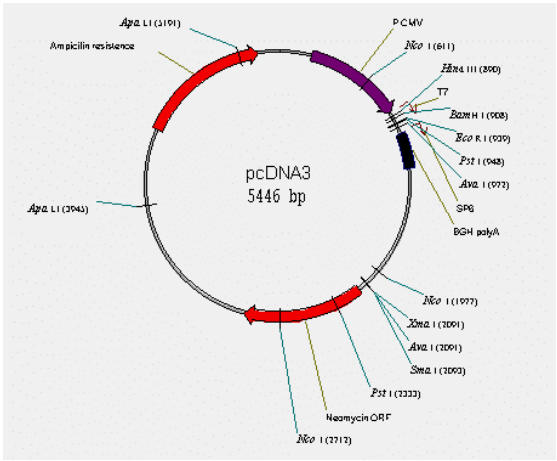

Figure 2.2 pcDNA3 vector.

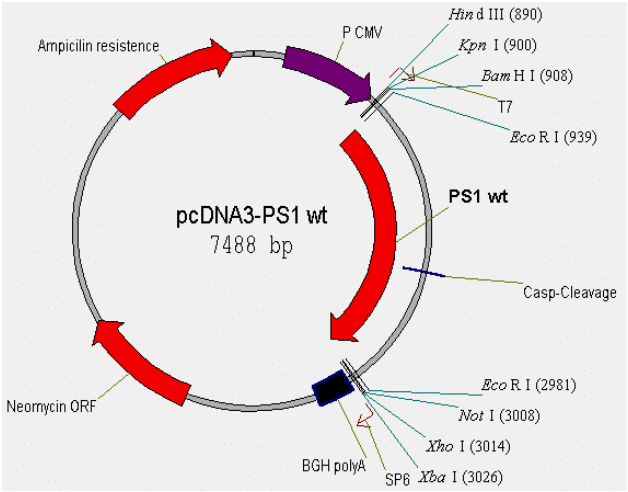

Figure 2.3 pcDNA3-PS1 vector. The cloned PS-1 cDNA is under the control of the CMV promotor, subsequent to a stop codon there is a polyA site. The vector contains a neomycin resistance for selection in eukaryotic cells, and an ampicillin resistance for selection in prokaryotes. All other vectors generated here are analogously. PcDNA3-PSCas contains the C-terminal PS1 Fragment after the caspase cleavage site.

2.3.2 Gelelektrophoresis

The characterization of DNA and RNA were performed by gelelectrophoresis, which allow the separation of charged molecules. Nucleic acids are negatively charged molecules, and are moved by electric current through a matrix of agarose towards the anode. The size of the fragments is determined using a marker. For the preparation of an 1% agarose gel, 1g agarose was dissolved in 100 ml 1X TAE buffer and heated until the solution becomes clear. Ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel solution was poured into a prepared gel casting tray and allow to solidify before samples with the appropriate loading dye were runned by electrophoresis at 50-150 volts. DNA was visualized by the UV-induced fluorescence of ethidium bromide dye intercalated into the nucleic acid. The amount of fluorescence is proportional to the amount of nucleic acid present. Dye bound to DNA has a much stronger fluorescence in UV light than free dye. UV light at 254 nm is absorbed by the DNA and transmitted to the dye and UV at 302 nm and 366 nm is absorbed by the dye itself. The energy is re-emitted at 590 nm in the red-orange part of the visible spectrum.

50x TAE-buffer:

-242 g Tris Sigma

-57,1 ml acetic acid	Merck
-100 ml 0,5 M EDTA (pH 8,0)	Merck
filled up with H_2O to 1l	
loading buffer: 10x BlueJuice	Invitrogen
Ethidiumbromid:	Invitorgen
agarose	Merck
100 bp DNA-ladder	New England Biolabs/Invitrogen
1 kb DNA-ladder	New England Biolabs/Invitrogen

2.3.3 Quantification of Nucleic Acid Concentration

The quantification of nucleic acid concentration was done by optical density measurements. The absorbance of UV light at 260 nm wavelength by nucleic acids gives an estimate of concentration, assuming firstly that there are no protein or phenol contaminants in the solution and secondly, that the concentration of the nucleic acid is greater than 250 ng / ml. The ratio of readings taken at 260 nm and 280 nm wavelengths (both in the UV range) gives an indication of the purity of the nucleic acid.

An OD unit corresponds to the amount of nucleic acid in µg in a 1 ml volume using a 1 cm path length quartz cuvette that results in an OD260 reading of 1.

For DNA OD260 1 = 50 µg / ml,

For RNA OD260 1 = 40 µg / ml,

For single stranded oligonucleotides OD260 1 ~ 33 mg / ml

2.3.4 RNA-Isolation

Total RNA from cells was isolated using the TRIZOL Reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, based on the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). To prevent introduction of RNases, all steps were worked under severe sterile conditions. Isolated RNA was dissolved in diethylpyrocarbonate (DEPC, 0,01%) treated and autoclaved water. 5X 10^6 cell were harvested, washed with PBS and lysed in 1 ml Trizol reagent. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After the addition of 0.2 ml of chloroform per tubes were vigorously shaked and incubate at room temperatue for 2 to 3 minutes. Samples were

centrifuge at 12,000 g for 15 minutes 4°C. After the centrifugation, the mixture is separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase containing RNA . The aqueous phase was transferred to a fresh tube and precipated by mixing with 0.5 ml isopropylalcohol. After an incubation at room temperature for 10 minutes samples were centrifuged at 12,000 $\cdot g$ for 10 minutes at 4°C. The supernatant was removed and the small RNA pellet washed with 1ml 75% ethanol. Following another centrifugation step (7,500 $\cdot g$ for 5 minutes at 4°C), the RNA pellet was briefly dried and dissolved in 50 µl DEPC treated water.

TRIZOL Reagent	Invitrogen
Chloroform	Merck
Isopropylalcohol	Merck
Ethanol	Merck
Diethylpyrocarbonate (DEPC)	Invitrogen

2.3.5 cDNA- Synthesis

Isolated RNA was used for first-strand cDNA synthesis reaction catalyzed by SuperScript. II Reverse Transcriptase (RT). This enzyme is not inhibited significantly by ribosomal and transfer RNA, it may be thus be used effectively to synthesize first- strand cDNA from a total RNA preparation. Oligo(dT), which hybridize to 3′ poly(A) tails, were used as primer. The following components were mixture for each individual sample before starting the cDNA synthesis reaction:

2 µg total RNA

10mM dNTPs mix	1 µl
Oligo(dT)12-18 (0.5 µg/µl)	1 µl
filled up with DEPC-treated water to	10 µl

The samples were incubate at 65°C for 5 min to destroy secondary structures, and then place on ice for at least 1 min.

The following master mixture was prepared:

10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNaseOUT (Recombinant RNase Inhibitor)	1 µl

Both, RNA/primer and master mixture were mixed and incubate at 42°C for 2 min. Finally, 1 µl (50 units) of SuperScript II RT was added to each tube and incubate at 42°C for 50 min. The reaction was terminated by an incubation step at 70°C for 15 min. At the end 1 µl of RNase H was added to each tube and incubate for 20 min at 37°C before proceeding to amplification of the Target DNA by PCR.

SuperScript First-Strand Synthesis System for RT-PCR	Invitrogen
$MgCl_2$	Invitrogen
DTT	Sigma

2.3.6 Reverse Transcriptase PCR (RT-PCR)

Following Target DNAs were amplificated with appropriate primer pairs by PCR for quantification:

Target	Primer	Product size	Annealing Temp
AIF, human	forward 5' -GGA TCC TGG GGC CAG GGT ACT GAT-3'		
	reverse 5‘ -CTC GGG GAA GAG TTG AAT CAC TTC-3’	550 bp	60 °C
ß-Actin, human	forward 5’-GAG CTA CGA GCT GCC TGA CG-3’		
	reverse 5’-GTA GTT TCG TGG ATG CCA CAG-3’	119 bp	58 °C
GSK3ß, human	forward 5‘ -ATT ACG GGA CCC AAA TGT CA-3’		
	reverse 5‘ -TGC AGA AGC AGC ATT ATT GG-3’	217 bp	55 °C
PS1, human	forward 5‘ -GCA CAG AAA GGG AGT CAC AAG-3’		
	reverse 5‘ -TAA TTG GTC CAT AAA AGG-3’	425 bp	46 °C
PS1Cter, human	forward 5‘ -GGTAAAGCCTCAGCAACAGC-3’		
	reverse 5‘ -AAACAAGCCCAAAGGTGATG-3’	153 bp	59 °C
AIF, rat	forward 5' -AGT GGA AGA CTG GCT GGA GA-3'		
	reverse 5‘ -TCA CTC TCC GAA CGG ATA-3’	217 bp	59 °C
Bax, rat	forward 5' -AGG ATC GAG CAG AGA GGA TG-3'		
	reverse 5‘ -GAG GAC TCC AGC CAC AAA GA-3’	449 bp	59 °C
Bcl-2, rat	forward 5' -CTG GCA TCT TCT CCT TCC AG-3'		
	reverse 5‘ -CTC ACT TGT GGC CCA GGT AT-3’	576 bp	55 °C
GAPDH, rat	forward 5' -GGCTGCCTTCTCTTGTGA-3'		
	reverse 5‘ -CTC GTG GTT CAC ACC CAT-3’	355 bp	57°C
GSK3ß, rat	forward 5' -GGA TCT GCC ATC GAG ACA TT-3'		
	reverse 5‘ -CCA ACT GAT CCA CAC CAC TG-3’	273 bp	58 °C

The following mixture was prepared for each individual PCR sample:

10X Tag buffer with Mg^{2+}	5 µl
5X Tag enhancer (heated to 65°C)	10 µl
dNTPs mix (10 mM each)	2 µl
Taq polymerase (5U/µl)	0,5 µl
Primer forward (100 pmol/µl)	0,5 µl
Primer reverse (100 pmol/µl)	0,5 µl
cDNA	2 µl
H_2O	29,5 µl
Total volume	50 µl

The PCR was run in a thermal cycler, with following adjusted cycling parameter:

10 min:	94 °C
1 min:	94°C
1 min:	appropriate anneling temperature
1 min:	72°C
10 min:	72°C
chilled to	4°C

The reactions were stopped in the exponential phase of amplification (After 20 or 25 cycles respectively), since at some later cycles the amplification rate drops to near zero (plateaus), and little more product is made. As control, actin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) standards were used. The PCR products were separated on a 1.5 % agarose gel, stained with ethidium bromide, and visualized under UV light.

Primer Ordering	MWG Biotech
10X Tag buffer (500mM KCL, 100mM Tris-HCL pH 8,3, 15 mM $Mg(OAc)_2$)	Epppendorf
5X Tag enhancer	Eppendorf
dNTPs mix (10 mM each)	Eppendorf/Invitrogen
Taq polymerase	Eppendorf/Invitrogen

2.3.7 Cloning of PS-Plasmids

Human PS1 in pcDNA3 was a kind gift from C.Haaß (München, Germany) to our laboratory. PS-1Cas was amplified from PS-1-pcDNA3 using *PfuI* polymerase and appropriate primers with an annealing temperature of 51°C. The resulting PCR fragments were subcloned into Kpn/XbaI restriction sites of pcDNA3 containing a gentamycin resistance gene. The ligation was performed with T4 DNA ligase overningt at 16°C. T4 ligase catalyzes the ligation of

double-stranded DNAs between the 3'-hydroxy and the 5'-phosphate termini in the presence of ATP:

1 µl pcDNA3-vector (50 ng/µl) (digest with Kpn/XBa
3 µl PSCas insert (50ng/µl)
0,5 µl Ligase
1 µl Ligase-buffer (10x)
4,5 µl H_2O
10 µl total volume

PSCas-Primer:	
Sense: 5'-CGG GGT ACC CCG ATG CCT CAT CGC TCT-3'	MWG Biotech
Antisense: 5'-TGC TCT AGA GCA CTA GAT ATA AA AT-3'	MWG Biotech
PfuI polymerase	New England Biolabs
T4 Ligase	New England Biolabs
Ligase buffer	New England Biolabs

Human PS1Mut (D345A) was constructed using theQuickChange™ mutagenesis kit from Stratagene. The vector PS-1-pcDNA3 was replicated with primers containing a single base exchange, leading to a directed mutant replication. GAC was exchanged with GCC in the sense-, GTC with GGC in the antisense-primer. Therefore, the amplificated vector contains a mutant aminoacid at site 345, substituting D (aspartate) with A (alanine). PCR was done with *PfuI* polymerase. Annealing temperature was 54°C, extension time 10 minutes. Parental DNA was digested with 10.000U *DpnI*, in order to remove methylated parental vector DNA and amplified DNA subsequently transformed with Top 10 component cells. Base exchange was approved by sequencing.

Psmut-Primer
Sense 5'-CCCAGAGGGCCAGTCATCTAGGG-3'
Antisense 5'-CCCTAGATGACTGGCCCTCTGGG-3'

QuickChange™ mutagenesis kit	Stratagene
PfuI polymerase	New England Biolabs

2.3.8 Transformation in competent Bacteria

For the amplification of plasmide DNA competent bacteria were transformed. The competent bacterial cells, pretreated with $CaCl_2$, were stored at –80°C. Therefore, first the competent cells (50 µl/tube) were thawed on ice and gently mixed with 10 µl plasmide DNA (10 ng).

After an incubation on ice for 30 min, cells were heat shocked (1 min at 42°C). Cells were placed on ice for 2 min and 250 µl SOC medium added. It followed a gently shaking at 37°C for 60 min and finally cells were plated on LB plus antibiotics plate. Plates were incubated in an inverted position in the 37°C incubator overnight. Individual clones were picked and cultured in 2 ml or 200 ml SOC-Amp medium for Plasmide DNA minipreparation and maxipreparation, respectively.

Top Ten, competent bacteria	Invitrogen
SOC medium:	
20 g LB-powder was dissolved in 1 l H_2O and autoclaved.	Invitrogen
SOC-Amp-medium:	
SOC-medium with 0,1 mg/ml Ampicillin-finalconcentration	
Ampicillin-Stock: 50 mg/ml	Merck
LB plus antibiotic plate:	
1 l autoclaved SOC-medium and 15 g Agar were heated to become a clear solution. After a chilling 1 ml Ampicillin (0,1 mg/ml finalconcentration) was added, and about 20 ml casted into a dish.	Invitrogen

2.3.9 Plasmid-Mini/Maxipreparation

The plasmid purification protocols are based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to anion-exchange resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer, and then concentrated and desalted by isopropanol precipitation.

Plasmide DNA was isolated from transformed bacteria suspension by mini preparation; the bacterial suspension was centrifuged at 12,000 · *g* for 10 minutes at 4°C. The bacterial pellet was completely resuspended in 0.3 ml of Buffer P1 containing RNase A. Buffer P2 (0,3 ml) was added, mixed gently by inverting the tube 4–6 times. After an incubation at room temperature for 5 min 0.3 ml of chilled Buffer P3 was added, mixed immediately and incubate on ice for 5 min. The precipitated proteins were separated by a centrifugation step at 18,000 · *g* for 10 minutes at 4°C. The clear supernatant was loaded onto a spin column in a 2 ml

collection tube and allow to enter the resin by gravity flow. After a centrifugation step (1 min) the spin column was washed twice with 0,75 ml buffer PE by centrifugation. Finally the column was placed in a new microfuge tube and the DNA eluated with 50 µl EB buffer. The isolation of plasmide DNA by maxi preparation was performed in the same procedure, only with differences in the used volumes. Afterwards DNA was precipitated with 0,7 volumes) of room-temperature isopropanol, centrifuged immediately at 12,000 x *g* for 30 minutes at 4°C min and the supernatant carefully decanted. The DNA was washed with 1 ml of 70% ethanol to remove precipitated salt, air-dried for 5 min, and redissolve in sterile water. For determination of the yield, DNA concentration was measured by UV spectrophotometry.

Resuspensionsbuffer P1: 50 mM Tris-HCl, pH 8,0 10 mM EDTA, 100 µg/ml RNase A	Qiagen
Lysisbuffer P2: 200 mM NaOH 1% SDS	Qiagen
Neutralisationbuffer P3: 3 M Natriumacetat, pH 5,5	Qiagen
Washing buffer PE: 1 M NaCl 50 mM MOPSD, pH 7,0 15% Isopropanol	Qiagen
Eluationsbufer EB: 1,25 M NaCl 50 mM Tris-HCl, pH 8,5	Qiagen
Isopropanol	Merck
Ethanol	Merck

2.3.10 Analytical Restrictionanalysis

Plasmide DNA was digest with restriction endonucleases for cloning, or to verify the successful ligation or transformation, thereby taking into account the optimal reaction buffer, incubation temperature or the BSA concentration. A typical preparation was incubated for 2 h at 37 °C as followed:

5 µl Plasmid-DNA	
1,5 µl NEB-Puffer 2	New England Biolabs
0,7 µl *Kpn*	New England Biolabs
0,7 µl *Xba*	New England Biolabs
1,5 µl BSA (10x)	New England Biolabs
10,6 µl H_2O	
20 µl total volume	

2.4 ANALYTICAL CELLBIOLOGICAL METHODS

2.4.1 Detection of cell viability

2.4.1.1. Induction of cell death

The detection of cell viabillity was performed by the following described methods. Cell death was induced by hydrogen peroxide, staurosporine or Fas-ligand.
Oxidative stress was induced with hydrogen peroxide (freshly prepared solutions) in PC12 and HEK cells. The respectives concentrations are stated below for each experiment. In other experiment staurosporine was used to induce apoptosis. Stauroporine is an alkaloid produced by *streptomyces* bacteria. The exact mechanism of its apoptosis inducing effect is still not known. An inhibition of protein kinase (Swannie et al. 2002) and cyclin-dependent kinase (Mikolajczyk et al. 2003, Gray N.1999) was reported. The use of staurosporine derivates in cancer therapie is discussed (da Rocha et al. 2002).
Jurkat cells were additionally incubated with Fas-ligand (50 ng/ml) for 24 hr. When announced, cells were incubated 1 to 3 hours prior to cell death induction with different cell permeable caspase inhibitors or SP600125, a selective anthrapyrazolone inhibitor of JNK.
The inhibitors were solved in DMSO.

Caspase 2 Inhibitor (Z-VDVAD-FMK)	Calbiochem/Merck Bioscience
Caspase 3 Inhibitor (AC-DEVD-CMK)	Calbiochem/Merck Bioscience
Caspase 6 Inhibitor (VEID-CHO)	Calbiochem/Merck Bioscience
Caspase 8 Inhibitor (IETD-CHO)	Calbiochem/Merck Bioscience
Caspase 9 Inhibitor (LEHD-CHO)	Calbiochem/Merck Bioscience
SP600125, anthra (1,9-*cd*) pyrazo-6-(2H)-one; 1,9pyrazoloanthrone	Calbiochem/Merck Bioscience
DMSO	Merck Bioscience
Hydrogen Peroxide	Sigma
Staurosporine	Merck Bioscience
Fas-ligand and potentiator kit	Upstate Biotechnology (Biozol)

2.4.1.2. Quantification of apoptosis by flow cytometry

Apoptosis was quantified by propidium iodide staining using a fluorescence-activated cell sorter (FACS). The principle of flow cytometry is follows: a beam of laser is projected through a liquid stream that contains cells, or other particles, which when struck by the focused light give out signals, which are picked up by detectors. These signals are then

converted for computer storage and data analysis, and can provide information about various cellular properties. The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. For the measurement of biological/biochemical properties, the cells are usually stained with fluorescent dyes which bind specifically to cellular constituents. The excitation of dyes by the laser beam leads to an emission of light at longer wavelengths which is pick up by detectors. Three fluorescent detectors (FL1, FL2 and FL3) are available in the FACS. All green (e.g. FITC) emissions are detected by FL1, all red by FL2 and all far red by FL3. In addition to these fluorescent emissions the FACS can also collect information on reflected laser light. There are two additional detectors that measure forward and right angle light scatter. Forward light scatter (FSC) measures that ability of a cell (or particle) to deflect light from its path which is a parameter for the cell size. Side scatter (SSC) measures the ability of a cell (or particle) to redirect light at a 90° angle, which is a parameter for cellular structure.

As apoptotic fluorescence dye propidium iodide was used, which is an analogous of ethidium bromide. It intercalates between the bases in double stranded DNA. When stacked between the bases, interactions between the π-electrons of the dye and the DNA bases occur, resulting in an altered emission maximum. The emitted fluorescence intensity is dependent on the length of the DNA molecule; the more basepairs the molecule consists of, the stronger is the fluorescence intensity subsequent to appropriate excitation. According to Nicoletti et al. (1991) apoptosis can be detected by lysing the cells in hypotonic buffer and staining of the DNA content. Sub-G_0-DNA content, indicative for apoptotic nuclei, is determined by flow cytometry in FL2.

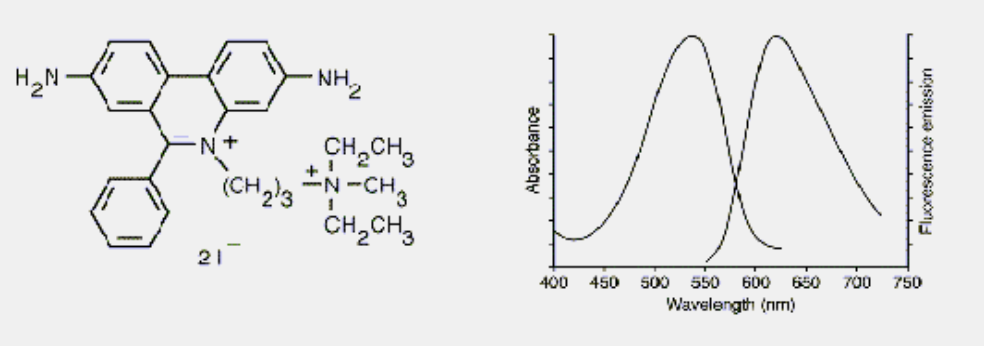

Figure 2.4. Molecular structure of propidium iodide. It posses the common planar conformation with conjugated bindings as the most DNA intercalating substances. Absorbance and emission spectra of propidium iodide.

Cells were harvested by centrifugation at various time points after cell death induction, washed with PBS and the pellets were resuspended in lysis buffer containing propidium iodide. Samples were stored at 4°C overnight before flow cytometry analysis using Cell Quest software. As shown in Fig. 2.5 vital cell populations display a very small percentage of apoptotic $subG_0$ nuclei (M1) and exhibits a huge G_0/G_1-peak. A smaller G_2-peak is also displayed in a histogram plot. Apoptotic cells display a higher percentage of $subG_0$ nuclei. The G_0/G_1-peak represents the cells in the G_0-, G_1-phase of the cell cycle, respectively. Doubled DNA contents owned by cells in the G_2-phase are displayed as G_2-peak. In between those two peaks, there is a population, displaying cells that are in the DNA-synthesis (S) phase.

Lysis buffer:

0.1% sodium citrate	Merck Biosciences
0.1% Triton X-1000	Sigma
50 µg/ml propidium iodide	Calbiochem/Merck Biosciences

Fluorescence activated cell sorter (FACS, flow cytometer) FACSCalibur™
CellQuest™ analysis software
FACSComp™ software
SimulTest™ software — all Becton Dickinson

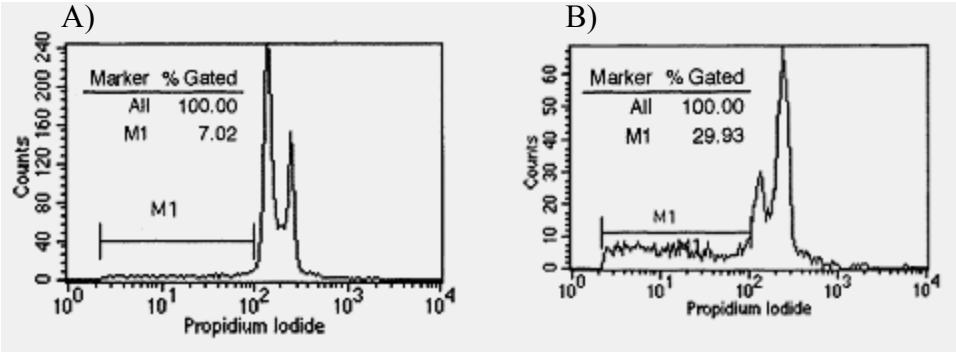

Figure 2.5 Representative histogram of PI-stained cells. A) Basal apoptotic levels, B) apoptotic stimulated cells.

2.4.1.3 MTT-assay

In some experiments the cytotoxicity was measured using the MTT assay kit. This assay which allow to measure metabolic activity is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH. The MTT formazan crystals are

insoluble in aqueous solution, but may be solubilized by adding the solubilization solution included in the kit. The solubilized formazan product is than photometrically quantified using an ELISA reader. An decrease in the number of living cells results in an decrease of total metabolic activity which leads to a weaker color formation. For the respective experiments cells were plated the day before at a density of 5 X 10^4 cells/well in 96-well tissue culture plate. After the indicated periods of time of cell death incubation (total volume 200 µl), MTT reagent was added (20 µl, final concentration 1.0 mg/ml) and incubation was continued for another 2 hrs. The formazan crystals were solubilized by adding 100 µl of a 20%SDS/50% N,N-dimethly-formamide solution (Mosmann, 1983). The absorption of the solubilized formazan was measured in triplicates at 570 nm using a microplate reader. MTT reduction values are expressed as percentages relative to control cells (= 100%).

Cytotoxicity Proloferation Kit (MTT-Assay)		Roche Diagnostic
MTT-Solubilzation solution:		
N,N-Dimethyl-formamide	90 ml	Sigma
Solubilization solution (from kit)	90 ml	
Sodium dodecyl sulfate	27g	Sigma
(soluble at 37 °C, stored at 4°C)		

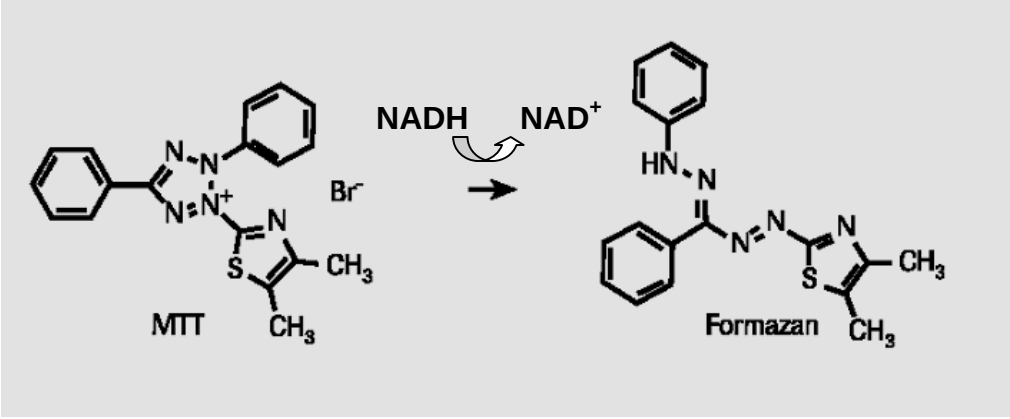

Figure 2.6. Metabolization of formazan salt by viable cells.

2.4.1.4 Caspase-Assay

The caspase-assay is a method, which allows to detect early events in apoptosis. While the quantification of apoptosis using propidium iodide using flow cytometry only includes cells holding a reduced DNA amount (sub G_0), the caspase assay detects the activity of caspases in cells, in which apoptosis is actually ocurring. The reduced DNA content is a consequence of fragmented DNA present in apoptotic vesicles. Caspase activity is needed for the cleavage of different substrates, like cytoskeleton or nuclear proteins involved in DNA metabolism and repair.

The assay is based on the cleavage of synthetic tetrapeptides tagged with a colorimetric or fluorogenic dye, which a used as caspase substrates. Since every caspase is specific to a cleavage site sequence, the activity was measured monitoring the release of para-Nitroaniline (*p*NA) from the tetrapeptide (see fig. 2.4) using a photometer (λ = 405 nm).

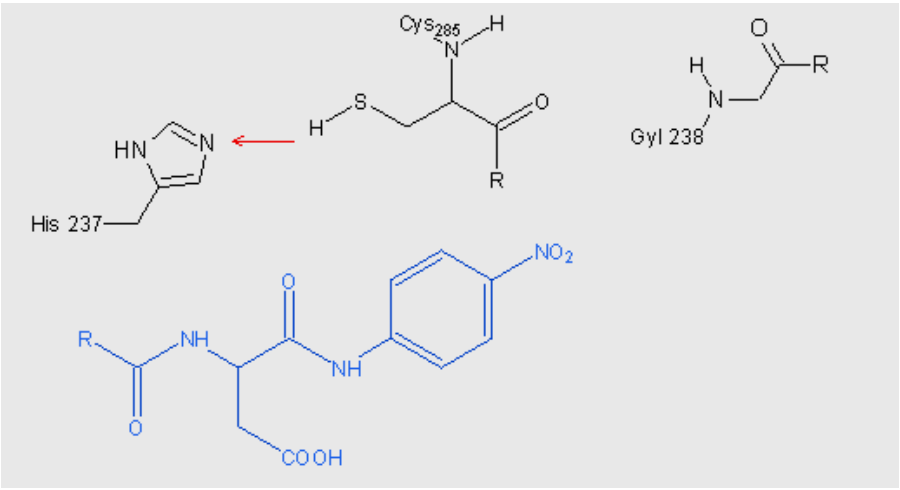

Figure 2.7. Catalytic center of caspase 3 according to Earnshaw et al. (1999). All caspases hydrolyze peptide bonds on the carboxyl side of an aspartate residue (termed the P1 residue).

The requirement for aspartate at the P1 position of a substrate and the catalytic residues are conserved in all human caspases. This aspartate is buried in a deep pocket, termed the S1 site which is lined by Arg179, Gln283, Arg341, and Ser347 of the enzyme. The specificity of caspases is due to structural feature. For example, in caspase 1 the S4 subsite is a large, shallow hydrophobic depression that easily accommodates bulky hydrophobic side chains. In caspase 3, this subsite consists of a rather narrow hydrophilic pocket that can accommodate small acidic side chains but excludes bulky side chains because of an occluding Trp.

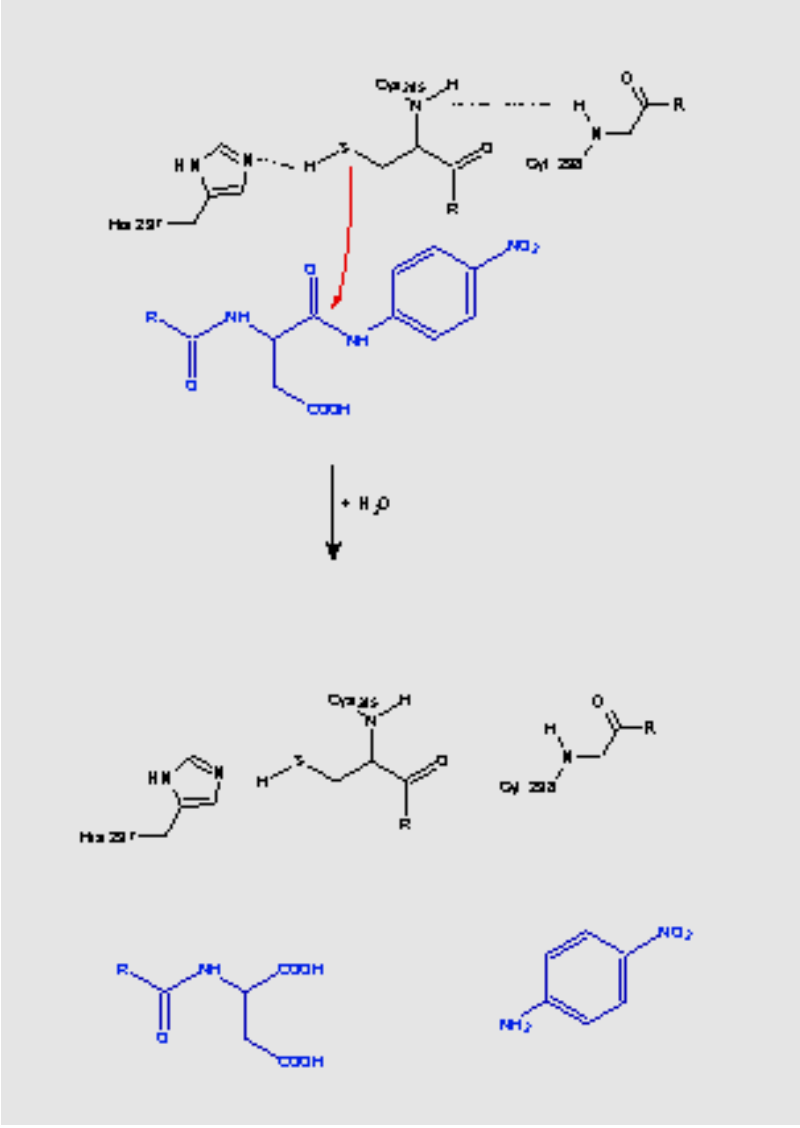

Figure 2.8. As shown for caspase 3 the substrate cleavage involves deprotonation of the sulfhydryl of Cys285 by the imidazole ring of His237, thereby yielding a thiolate nucleophile that attacks the carbonyl carbon of the scissile bond to form a tetrahedral intermediate, which is stabilized by hydrogen bonding to the amide nitrogen of Gly238.

Cell Extract Preparation:

For the measurement of the caspase activity cells were plated the day before at a density of $5x10^6$ cells/culture dish (∅ 10 cm). After treatment with hydrogen peroxide or staurosporine cells were harvested, and after a centrifugation step (5 min, 300 g) the culture medium aspirated. The cells containing pellet was washed with PBS and lysed in 100 µl lysis buffer (10 mM HEPES, 0.1% Triton X-100, 1 mM PMSF, 0.1 mM EDTA, 1 mM DTT, 1 µg/ml pepstatin A, 1 µg/ml leupeptin, 0.1 % CHAPS, pH 7.4) and incubated for 30 min on ice. Lysates were centrifuged at 20,000 x *g* for 10 min at 4°C, and the protein amount of the supernatant determined by a Lowry protein test (see 2.4.2.1). For later use, extracts were stored at –70°C.

Cell Lysis -Buffer (pH 7.4)	
10 mM HEPES	Sigma
1 mM DTT	Sigma
0,1 mM EDTA	Sigma
0,1% CHAPS	Sigma
0,1% Triton X100	Merck
1 mM PMSF	Sigma
1 µg/ml Pepstatin	Sigma
1 µg/ml Leupeptin	Sigma

Photometric monitoring:

Caspase activity of the probes was measured at room temperature and λ = 405 nm. In a total volume of 100 µl, 80 µl assay buffer and 10 µl cell extract were mixed and incubated for 10 min. The reaction was started by adding 10 µl of the colorimetric caspase substrate (Ac-DEVD-pNA, Ac-VEID-*p*NA, Ac-VDVAD-*p*NA, Ac-IETD-*p*NA Ac-LEHD- *p*NA for caspase 3, caspase 6, caspase 2, caspase 8 and caspase 9, respectively; final concentration of 200 µM). The absorbance A_{405} was measured for 30 min and the slope of the A_{405} nm vs. linear time course plot calculated. As controls, assay mixtures containing the respective recombinant enzyme, inhibitor or no cell extract were used.

Assay-buffer (pH 7.4)	
100 mM NaCl	Merck
50 mM HEPES	Sigma
10 mM DTT	Sigma
1 mM EDTA	Sigma
10 % Glycerol	Merck
0,1 % CHAPS	Sigma

Substrates:

Caspase-3-substrate (Ac-DEVD-*p*NA, colorimetric)	Calbiochem
Caspase-6-substrate (Ac-VEID-*p*NA, colorimetric)	Calbiochem
Caspase-2-substrate (Ac-VDVAD-*p*NA, colorimetric)	Calbiochem
Caspase-9-substrate (Ac-IETD-*p*NA, colorimetric)	Calbiochem
Caspase-9-substrate (Ac-LEHD-*p*NA, colorimetric)	Calbiochem

Sample Activity Calculation

For the calculation of the sample activity the conversion factor was determined. 100 µl of *p*-Nitroaniline [50 µM *p*NA in assay buffer] were pipetted in to three wells and the absorbance measured. The calculation of the conversion factor is based on the concentration of *p*-Nitroaniline in the calibration standard (50 µM). The extinction coefficient for *p*-Nitroaniline in assay buffer is 10,500 M^{-1} cm^{-1} .

Conversion factor = 50 µM/ Absorption avarage (λ = 405)

The activity of a single probe is calculated using the slope (graph of blot absorbance vs. time course) and the assay volume:

activity (pmol/min) = slope (DA/min) x conversion factor x assay vol (100 µl)

p-Nitroanilin	Calbiochem

2.4.1.5 Determination of mitochondrial membrane potential

Cells were plated the day before at a density of $2x10^5$ cells/well in a 24 well plate. In respective experiments, cells were pretreated for 1 or 3 hr with JNK- or different caspase inhibitors and hydrogen peroxide was added for 6 or 24 h (total volume in well: 500 µl). The membrane potential of the inner mitochondrial membrane was measured using the dye Rhodamine 123. Rhodamine 123 is widely used as a structural marker for mitochondria and as an indicator of mitochondrial activity. The dye was added to the cell culture medium at a concentration of 0.4 µM for 15 min. The cells were washed twice with HBSS (500 µl) and the fluorescence was determined with a fluorescence reader (filters: λ = 495 nm for absorption, and λ = 535 nm for fluorescence). Transmembrane distribution of the dye depends on the mitochondrial membrane potential ($\Delta\Psi_m$).

Rhodamine 123, Xanthylium, 3,6-diamino-9-(2-(methoxycarbonyl) phenyl, chloride	Molecular Probes

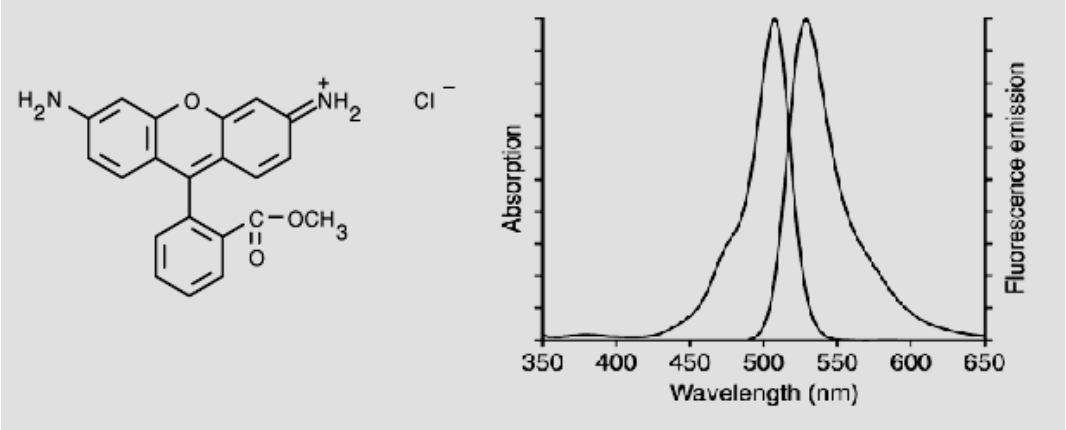

Figure 2.9. Molecular structure, and spectra of absorbance and emission of Rhodamine 123.

2.4.1.6 Determination of ATP-levels using a bioluminescence assay

ATP levels were determined using a bioluminescence assay (ViaLight^{TM} HT) kit from Cambrex/USA. The bioluminescent method utilises an enzyme, luciferase, which catalyses the formation of light from ATP and luciferin according to the following reaction:

$$\text{ATP + Luciferin + } O_2 \xrightarrow[\text{Mg++}]{\text{Luciferase}} \text{Oxyluciferin + AMP + PPi + } CO_2 \text{ + LIGHT (Emission at 565 nm)}$$

The emitted light is linearly related to the ATP concentration and is measured using a luminometer.

For the assay, HEK cells were plated the day before at a density of 5x10^4 cells/well in a 96 well white walled microplate. After 30 min of hydrogen peroxide incubation (total volume of 100 µl) 100 µl of ATP Monitoring Reagent was added to each well. The concentrations of ATP/ 5x10^4 cells were determined using a concentration dependent ATP standard.

ViaLight^{TM} HT bioluminescence assay Cambrex/USA

2.4.2 Proteinchemical methods

2.4.2.1 Determination of protein amount by Lowry protein test

The Lowry procedure is one of the most widely-used protein assays, being first described in 1951 (Lowry 1951). Under alkaline conditions, copper complexes with protein. When Folin-phenol reagent (phospho-molybdic-phosphotungstic reagent) is added, the Folin-phenol reagent binds to the protein. Bound reagent is slowly reduced and changes color from yellow to blue.

For the measurement, 5 µl of the probes, standart or the corresponding buffer as blank value were mixed with 25 µl of Bio-Rad reagent A (alkaline copper tartrate solution). Afterwards 200 µl of Bio-Rad reagent B (Folin-Ciocalteu's phenol reagent, 2N) was added. As standart a dilution series of Bovine Serum Albumin (BSA) were used. After an incubation of 15 min under weak agitation the absorbtion of the solution was measured at λ = 620 nm using a microplate reader. If reducing agents or detergents were present in the probes, first 20 µl of the Bio-Rad reagent S (surfactant solution) were added to 1 ml reagent A.

Bio-Rad DC Protein reagent A	BioRad
Bio-Rad DC Protein reagent B	BioRad
Bovine serum Albumine	Merck Biosciences

2.4.2.2 Western blotting

PC12 or HEK cells were plated at a density of $5x10^6$ cells/culture dish (∅ 10 cm) and treated with H_2O_2 for the indicated periods of time. Cells were harvested and lysed in 100 µl SDS sample buffer: Tris-HCl (62.5 mM, pH6.8); 2 % SDS, 10 % Glycerol, 50 mM DTT, 0.01 % bromphenol blue. After sonicating (1 min) and boiling at 95°C (5 min), proteins were separated by electrophoresis on a polyacrylamide gel (12-18 %). The separated proteins were transferred to a PVDF membrane. These membranes were stained with Ponceau S red (reversible stain) to visualize the proteins. Nonspecific sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline, 0.1% Tween-20 (TBST) for 1hr. The incubation of the membranes with Primary Antibody (at the appropriate dilution) was performed in primary antibody dilution buffer (1X TBS,0.1%Tween-20 with 5%BSA) with gentle agitation overnight at 4°C. Proteins were visualized with the appropriate secondary antibodies (1 hr

incubation at appropriate dilution in 1X TBS, 0.1% Tween-20 with 5% nonfat dry milk at room temperature). After thorough washing with TBST, membranes were covered with ECL™ detection reagents and quickly exposed to an autoradiography film. Membranes were routinely stripped for actin control.

Antibodies:	
Caspase 3 (cleaved, Asp 175), human, rat, mouse	Cell Signaling Technology
Caspase 9, rat	Cell Signaling Technology
Caspase 2, human, rat, mouse	Alexis
Caspase 8, human, rat, mouse	Biocat, Heidelberg
Phospho SEK/MKK4 (Thr 261)	Cell Signaling Technology
Phospho JNK (Thr183/Tyr185)	Cell Signaling Technology
Phospho-c-Jun (Ser63)	Cell Signaling Technology
Actin, human, rat, mouse	Santa Cruz-Biotechnology
Secondary Antibodies:	
Anti-Rabbit IgG, Peoxidase Conjugated	Calbiochem
Anti-Rat IgG, Peroxidase Conjugated	Calbiochem
Anti-Goat IgG, Peroxidase Conjugated	Calbiochem
ECL-reagent	Amersham
Running buffer:	
25 mM Tris-HCl, pH 8.3 at RT,	
192 mM glycine	
0.1% (w/v) SDS.	all Merck or Sigma
Stacking gel :	
40% acrylamide	Bio-Rad
2% bis-acrylamide	Bio-Rad
Tris-HCl, pH 6.8	
10% SDS	
H_2O	
TEMED	
APS	all Merck or Sigma
Running gel:	
40% acrylamide	Bio-Rad
2% bis-acrylamide	Bio-Rad
Tris-HCl, pH 8.8	
10% SDS	
H_2O	
TEMED	
APS	all Merck
Transfer buffer:	
12 mM Tris-HCl, pH 8.3 at RT,	
96 mM glycine,	
20% (v/v) methanol.	all Merck or Sigma
Chill to 2-8°C prior to use.	

2.4.2.3 Immunoblotting of cytoplasmic and mitochondrial proteins

To separate proteins in cytoplasmic and mitochondrial fractions a digitonin permeabilization according to Gottlieb et al. (2002) was performed. 5 x 10^6 cells were esposured to oxidative stress for different periods of time. After washing with ice-cold PBS cells were resuspended in permeabilization buffer containing 75 mM NaCl, 1mM NaH_2PO_4, 8 mM Na_2PO_4, 250 mM Sucorse, 1mM PMSF, additional protease inhibitors and 0,05% digitonin. Following a centrifugation step at 800xg at 4°C for 10 min, the supernatant was separated from the pellet consisting of mitochondria and cellular debris. The supernatant containing cytoplasmic proteins was purified by centrifugation at 13,000 x g at 4°C for 10 min. The pellet consisting the mitochondrial fractions was lysed in a Triton lysis buffer (0,1% Triton in PBS). After 30 min on ice and a centrifugation step at 12.000 x g and 4°C for 5 min, the supernatant was used for immunoblotting. Equal amounts of protein (10 µg) of cytoplasmic and mitochondrial fraction were loaded on an arcylamid gel, separated by SDS-PAGE and blotted as described in 2.4.2.2.

2.4.2.4 Western Blot Stripping

To facilitate a 2nd round reprobing, membranes were kept sealed and moist at 4°C after the first ECL development. Membranes were incubated in stripping buffer at 50°C for 30 minutes. Afterwards membranes were washed thoroughly for multiple times with TBST. The washed membranes were than blocked and blotted as normal.

Stripping buffer:
62,5 mM Tris, pH 6,7
2 % SDS
100 mM Mercaptoethanol (freshly added) all Merck or Sigma

2.4.3. Genomic DNA electrophoresis (DNA ladder)

Apoptotic cell death was induced by reduction of trophic support at the indicated period of times. 5 x 10^6 cells were centrifuged at 300 x g, the pellet washed with PBS and incubated at 55°C overnight in 300 µl lysis buffer (10 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1 % SDS, 100 µg/ml Proteinase K). After adding RNase (10 µg/ml) the lysates were further incubated for 2 h at 37°C. DNA was extracted with Phenol/Chloroform, precipated with isopropanol, washed with ethanol and dissolved in Tris-EDTA buffer. Electrophoresis was carried out on 3% agarose gel containing ethidium bromid for visualization under UV light.

Lysis buffer for DNA-ladder:	
10 mM TRIS (pH 8,0)	
100 mM NaCl	
100 mM EDTA	
1% SDS	
100 µg/ml proteinase K	all Merck
RNase A	Sigma
phenol (equilibrated with TRIS buffer pH 7.4)	Sigma
chloroform	
ethanol	
agarose	all Merck
ethidium bromide solution (10 mg/ml)	Sigma

2.5. STATISTICAL EVALUATION OF THE DATA

The statistical calculations were performed using Prism 3®-Software (GraphPad Software, Inc, San Diego, USA). Data are given as mean ± SEM. For statistical comparison, paired *t*-test, student's *t*-test or one-way ANOVA followed by Tukey's posthoc test or two-way ANOVA were used. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1 CELL DEATH CASCADES IN A CELL MODELL EXPRESSING MUTANT APP REFELECTING LOW Aß LOAD

3.1.1 Characterization of PC12 cell lines overexpressing APPwt and APPsw

To investigate the effects of APP overexpression in oxidative stress-induced apoptosis several clones of PC12 cells were stably transfected with APP cDNAs. Similar expression of human APP in the investigated cell lines was confirmed by western blotting by Astrid Bonert from our lab. The human-specific antibody does not recognize any APP species in vector-transfected PC12 cells, while equal expression levels of human APP were detected in clones of APPwt cells and in clones of cells containing the Swedish mutation. Culture supernatants of the APP transfected cells were also analysed for Aß(1-40) production revealing four- to fivefold elevated Aß(1-40) levels in clones of APPsw cells compared to clones of APPwt cells (Fig. 3.1).

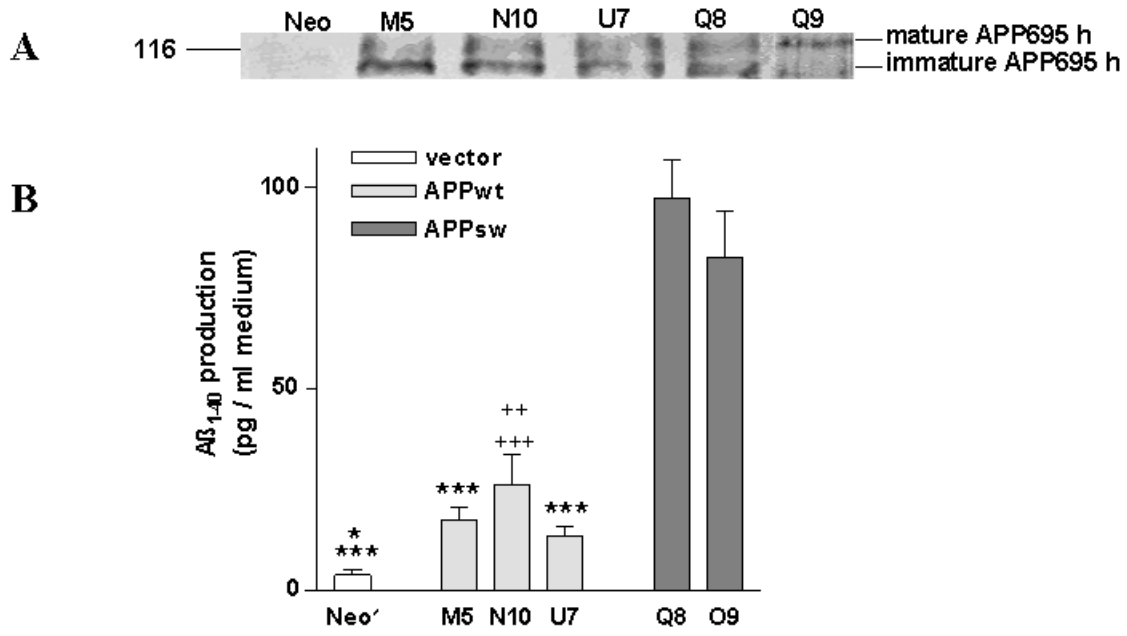

Figure 3.1 (A) Expression of human APP in transfected cell lines: Representative western blot: neo, PC12 vector transfected cell line; wtM5, wtN10, wtU7, PC12 clones stably transfected with pCMV695wt; swQ8, swO9, PC12 clones stably transfected with pCMV695sw. The higher molecular weight band differs from the posttranslational modification of APP. Human APP expression was similar in APPsw and APPwt clones.

*(B) Analysed conditioned media for Aß production: The clones expressing the Swedish mutant APPsw showed a four- to fivefold increase in the Aß(1– 40) production when compared to the APPwt clones (ANOVA: ***p<0.001, **p<0.01 vs. swO9 and swQ8, +++p<0.001 vs. swQ8, ++p<0.01 vs. swO9 posthoc Tukey's). The increase in the production of Aß(1– 40) was confirmed in three independent experiments for each clone. PC12 cells bearing human APPwt showed only a two- to threefold increase in Aß production compared to vector-transfected PC12 cells (ANOVA: * P < 0.05, * P < 0.05 vs. wtM5, wtN10 and wtU7, posthoc Tukey's).*

3.1.2 APPsw mutation leads to an enhanced vulnerability to oxidative stress induced apoptosis

Apoptosis was induced in transfected PC12 cells using the oxidative stressor hydrogen peroxide. Nuclear DNA fragmentation was quantitatively detected by propidium iodide staining and flow cytometry. No differences in apoptotic cell levels have been detected in basal apoptosis between APPsw-, APPw-, or vector-transfected clones (Eckert 2001).

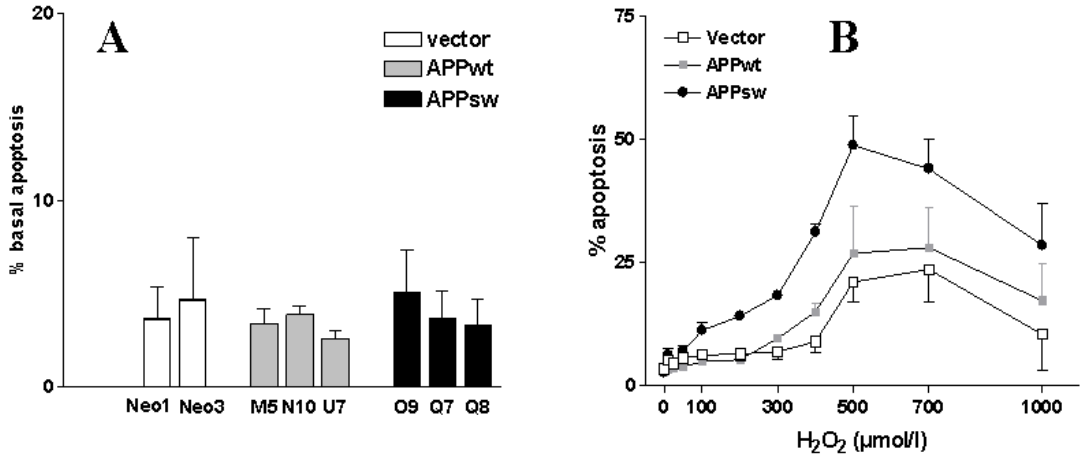

Figure 3.2 (A) Basal levels of apoptotic cells were not different between APPsw clones (swQ7, swQ8, swO9), APPwt clones (wtM5, wtN10, wtU7) and empty vector-transfected (NEO1, NEO3), PC12 cells. Data are means 6 SEM from 8–12 cultures in four to six independent experiments per clone. (B) Increase in apoptotic cells after treatment with increasing concentrations of H2O2. APPswPC12 cells showed enhanced sensitivity to oxidative stress-induced death compared to APPwt and vector cells (ANOVA, P , 0.01). Values are means 6 SEM from three or four independent experiments.

Importantly, clones bearing the APPsw mutation are sensitised to an exposure to oxidative stress for 24 hr in a concentration dependent manner with a maximum increase of apoptotic cells at a concentration of 500 µM hydrogen peroxide (Eckert 2001). Treatment with hydrogen peroxide led to a significantly enhanced maximum increase of apoptotic cells in APPsw-bearing PC12 cells (51,4% ± 5,3%) compared to APPwt-bearing cells (28,4% ± 4,7%) and vector control cells (21,3% ± 4,3%). Different clones with the same transfectant behave similarly in their sensitivity to H_2O_2-induced cell death (Fig. 3.2).

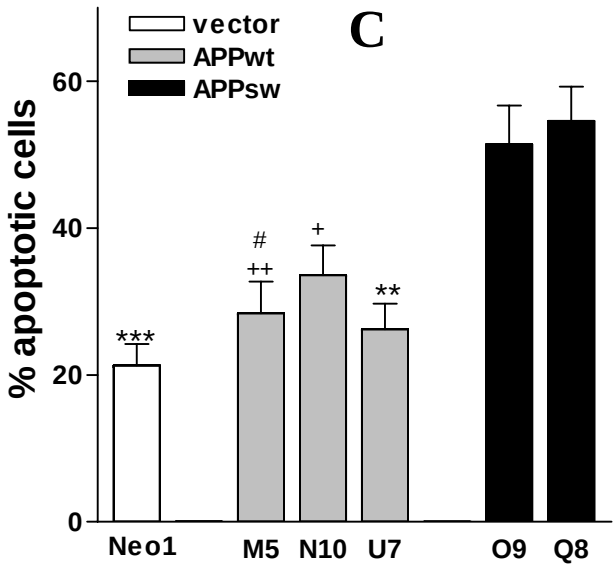

*Figure 3.2 (C) Increase in apoptotic cells after treatment with H_2O_2 in different clones from each cell line. Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. APPsw PC12 cells showed enhanced sensitivity to oxidative stress-induced death compared to APPwt and vector cells (ANOVA, P < 0.001). Different from pCMV695sw PC12 cells: posthoc Tukey's, ***p<0.001, **p<0.01 vs swO9 and swQ8, ++p<0.01, +p<0.05 vs. swQ8, #p<0.05 vs swO9. Values are means ± SEM from four to six independent experiments.*

3.2 CASPASE-ACTIVATION IN OXIDATIVE STRESSINDUCED CELL DEATH

All following experiments were performed with APPsw clone Q8 and APPwt clone M5.

3.2.1 Oxidative stress induces activation of caspase 2 in PC12 cells

Oxidative stress in the human brain has been implicated as one major cause of neuronal cell loss in AD patients. However, the exact mechanism still remains unknown. Aß appears to bind red

ox-active metals like zinc, copper or iron with high-affinity, resulting in production of hydrogen peroxide and auto-oxidation of the metalopeptide complex (Rottkamp 2001, Tabner 2002). The signalling cascades activated following the oxidative stress have not been widely studied. Therefore, the implication of caspase 2 was studied by measuring the activity and by western blotting. Cleavage of the photometric substrate Ac-VDVAD-p-nitroanilide by cytosolic protein extracts indicates the presence of caspase 2 protease activity in the cultures after exposure to hydrogen peroxide. The activity was already early induced in APPsw cells within two hours of induction (Fig. 3.3). Caspase 2 activity was continuously elevated over time compared to APPwt and vector-transfected cells. Maximum caspase 2 activity was measured after 2 hr, with a fourfold higher activity in APPsw cells than in APPwt and vector PC12 cells, respectively (Fig. 3.4).

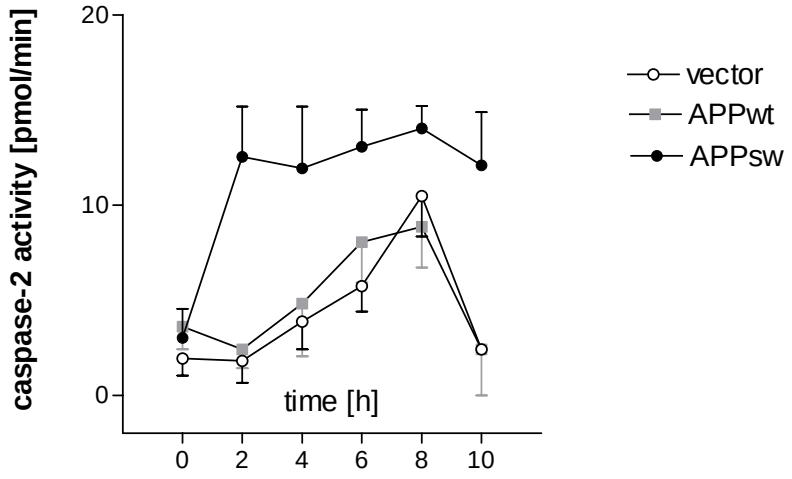

Fig. 3.3 Time course of caspase 2 activity in protein extracts from PC12 cells after treatment with 250 µM H_2O_2. Over the whole time period, APPsw bearing cells exhibit a significantly increased caspase 2 activity compared to APPwt transfected cells and vector controls: two-

way-ANOVA revealed significant differences between cell lines (P< 0.001) and time course (P< 0.001). Data are means ± SEM from 4-6 independent experiments.

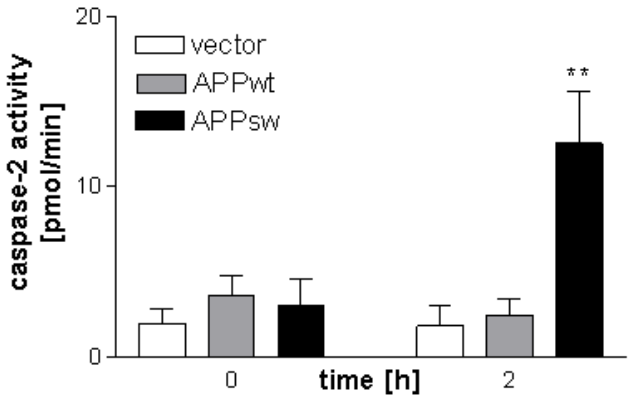

*Fig. 3.4 The caspase 2 activity was early induced in APPsw cells within two hours of oxidative stress induction and fourfold higher compared to APPwt and vector controls (**P< 0.01 vs. APPwt and vector, ANOVA). Data are means ± SEM from 4-6 independent experiments.*

The mechanism of activation of caspases occurs by a sequential cleavage of the zymogen to release the large and the small cleavage products. In accordance to the findings from the determination of activity, western blotting confirmed cleavage of caspase 2 within two hours of hydrogen peroxide induction. Again, the activation of caspase 2 was noticeable higher in APPsw cells compared to vector transfected controls.

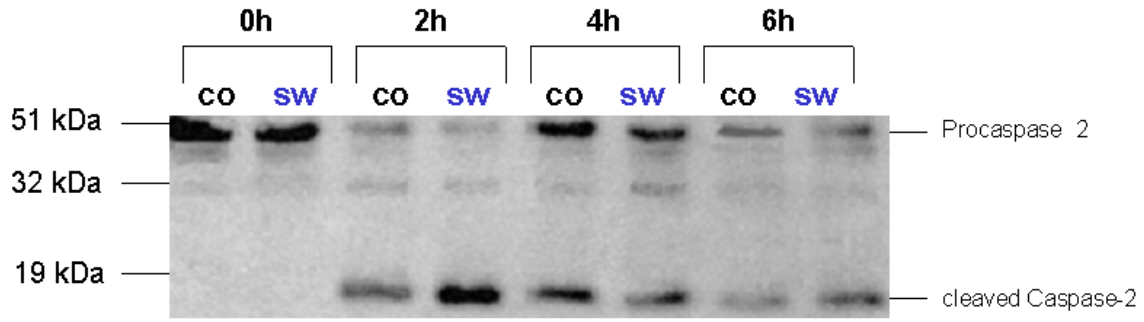

Fig. 3.5 Western blot analysis of caspase 2 activation in cell lysates of H_2O_2-treated PC12 cells. Increased expression of cleaved caspase 2 in APPsw cells is already detectable after 2 hr treatment. Representative Western blotting is shown from at least three independent observations.

3.2.2 Activation of caspase 8 in response to oxidative stress

Apoptosis associated with the Fas/Tumour Necrosis Factor Receptor (Fas/TNFR) family of death receptors requires caspase 8 activity and adaptor proteins such as FADD. The involvement of caspase 8 in processing of APP during apoptosis by caspase 8 has been reported, recently (Pellegrini 1999). The blocking of neuronal death by caspase 8 inhibitor IETD-fmk in Aß-induced cell death was also demonstrated (Ivins 1999). Hence, the activation of caspase 8 was anyalyzed in transfected PC 12 cell lines overexpressing intracellular high levels of Aß in order to ascertain the involvement of this initiator caspase in oxidative stress. Ac-IETD-*p*NA was utilized to measure caspase 8 activity in lysates of treated cells. In parallel to the early activation of caspase 2, caspase 8 activity increased about threefold after 2 hr of stress induction in PC12 cells expressing the Swedish double mutation and was significantly enhanced in a time-dependent manner compared to vector transfected controls (Fig. 3.6).

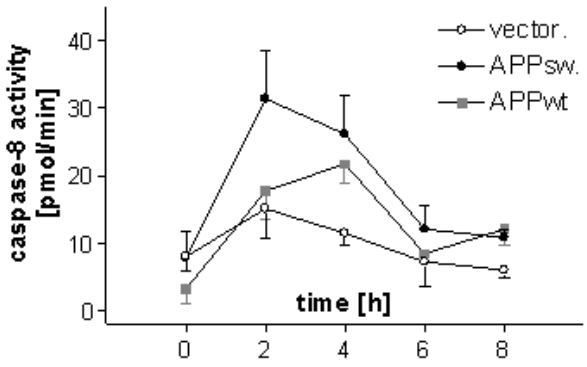

Fig. 3.6 Time course of caspase 8 activity in protein extracts from PC12 cells after treatment with H_2O_2. Data are means ± SEM from 4-6 independent experiments.

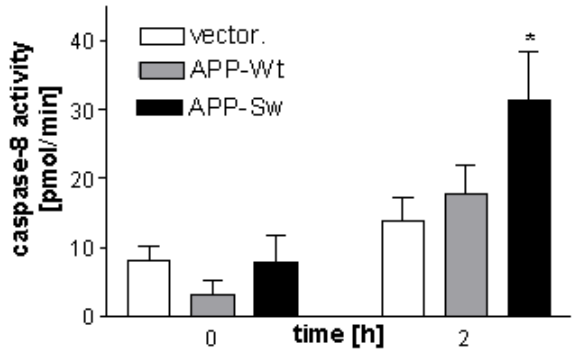

Fig. 3.7 The maximal caspase 8 activity was measured after 2 hr induction. APPsw bearing cells exhibit a significantly enhanced activity compared to vector controls (P<0.05 vs. vector, ANOVA). Data are means ± SEM from 4-6 independent experiments.*

Activation of caspase 8 involves a two-step proteolysis; the cleavage of the procaspase 8 to generate a 43 and a 12 kDa fragment which is further processed to 10 kDa. The large fragment is then cleaved to yield p26. Here, we used an antibody recognizing the 26 kDa large fragment of caspase 8. Western blot analysis showed a notable expression of active caspase 8 even in the untreated APPsw PC12 cells. The caspase 8 expression in APPsw cells was continuously higher compared to vector control during the induction of 250 µM hydrogen peroxide. (Fig. 3.8).

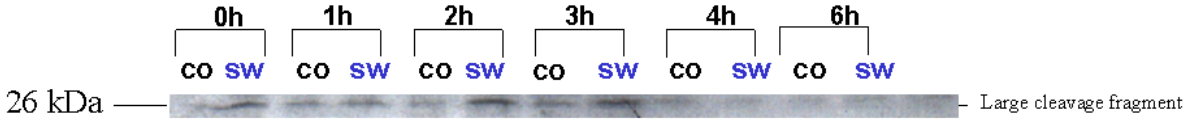

Fig. 3.8 Over the whole time period, the protein expression in APPsw cells was continuously higher compared to vector control as detected by an antibody recognizing the 26 kDa large cleaved fragment of caspase 8. Representative Western blotting is shown from three independent observations.

3.2.3 Involvement of caspase 9

Mitochondria play a prominent role in the intrinsic apoptosis pathway. During mitochondrial dysfunction, several essential players of apoptosis, including procaspases, cytochrome C, and apoptosis-inducing factor (AIF) are released into the cytosol. The multimeric complex formation of cytochrome C, APAF-1 (apoptotic protease-activating factor-1) and caspase 9 activates downstream caspases leading to apoptotic cell death. Therefore, it was examined whether oxidative stress in PC12 cells activates caspase 9 by using a polyclonal antibody specific for the inactive full length pro-caspase 9 and for the cleaved fragments of the protein. A time-dependent increase was detected in cleaved fragments of caspase 9 after treatment of the cells with hydrogen peroxide (Fig. 3.9). APPsw PC12 cells showed an elevated expression of the active cleaved fragments of caspase 9 compared to vector-transfected cells. A time-dependent cytochrome C increase in the cytosol has also been detected by western blot using a specific antibody (Marques 2003). The highest cytochrome C amount in the cytosolic fraction was detected after 4 hr oxidative stress induction.

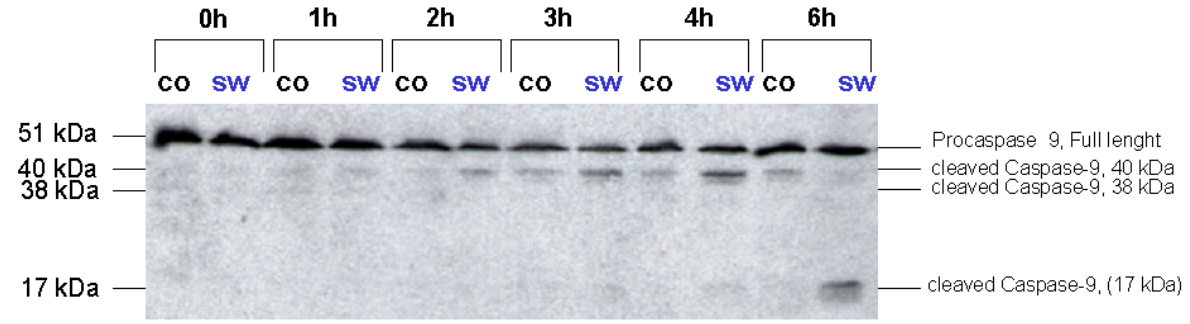

Fig. 3.9 Western blot analysis of caspase 9 activation in cell lysates of H_2O_2-treated PC12 cells. Representative Western blotting is shown from at least three independent observations.

In addition, caspase 9 activities were measured colorimetrically using Ac-LEHD-*p*NA as substrate in lysates of hydrogen peroxide-treated cells (Fig. 3.9). An early induced activation of caspase 9 after 2 hr was found, while the activity was increased in the APP-transfected cells compared to vector-transfected controls, but with no difference between APPsw and APPwt cells (Fig. 3.10).

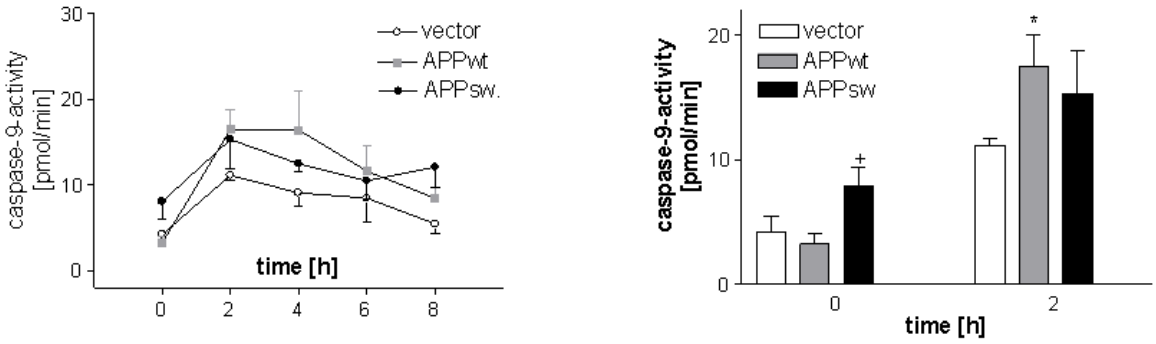

*Fig. 3.10 Enhanced caspase 9 activity in cell lysates from APP-transfected cells compared to vector transfected controls (*P < 0.05 vs. vector after 2 hr induction; $^{+}$P < 0.05 vs. vector and wt under basal conditions). Data are means ± SEM from 5-7 independent experiments.*

3.2.4 Increased caspase 3 activity of APPsw PC12 cells in response to oxidative stress

Furthermore, extracts from hydrogen peroxide-treated PC12 cells were prepared and measured for their ability to cleave the colorimetric substrate to Ac-DEVD-*p*NA, which is specifically cleaved by caspase 3. Cellular extracts from untreated control cultures showed very low Ac-DEVD-*p*NA cleavage in all cell lines (Fig. 3.11). Under these baseline

conditions, no significant differences could be detected between vector-, APPwt-, and APPsw-transfected PC12 cells. After exposure to oxidative stress, caspase 3 activity increased gradually and reached a maximum activity after 6 hr incubation, demonstrating a delayed increase as compared to the initiator caspases 2 and 8. APPsw and APPwt PC12 cells showed a significantly enhanced caspase 3 activity compared to vector-transfected clones with the strongest increase in the APPsw cells (Fig. 3.11).

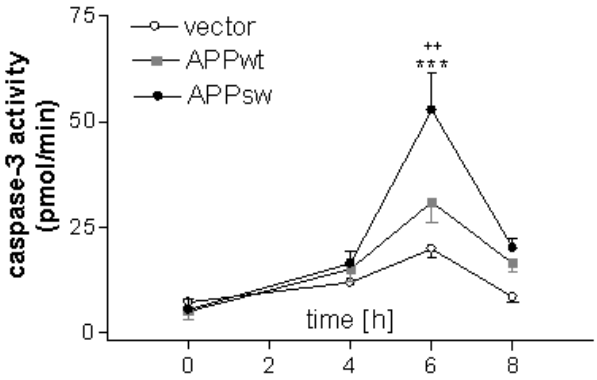

Fig. 3.11 Time course of caspase 3 activity in cytosolic protein extracts from H_2O_2-treated PC12 cells. Maximal caspase 3 activity was reached after 6 hr determined by DEVD cleavage. APPsw cells showed enhanced caspase 3 activity after oxidative stress-induced death compared to APPwt and vector cells. Maximal caspase 3 activity was reached after 6 hr of exposure to H_2O_2 ($^{++}P < 0.01$ vs. APPwt; $***P < 0.001$ vs. vector, ANOVA). Data are means ± SEM from 5-6 independent experiments.

By western blotting, we also found a significantly increased protein expression of the active caspase 3 in APPsw PC12 cells compared to control cells. The highest protein expression was evidenced after 6 hr H_2O_2 exposure by densitometric immunoblot analysis (Fig. 3.12).

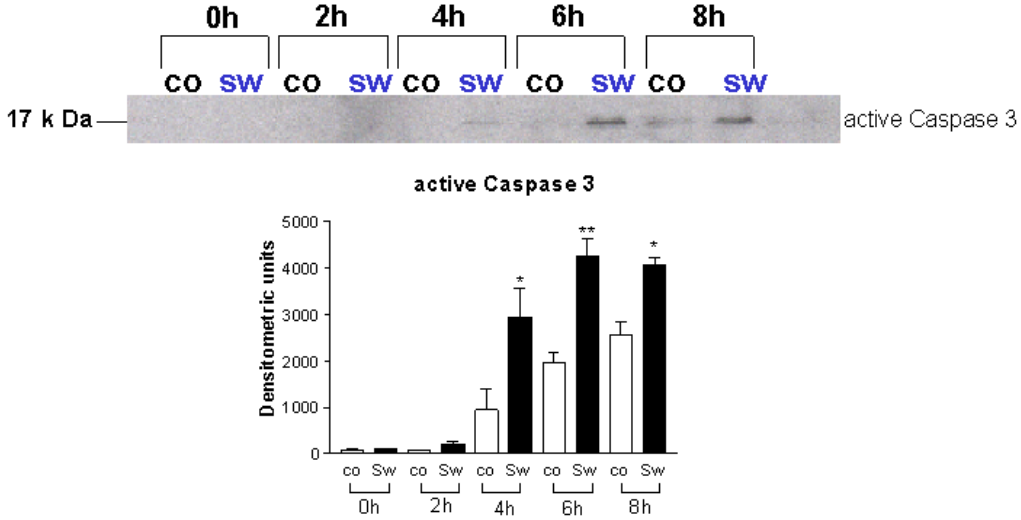

Fig. 3.12 Time course of western blot analysis of active caspase 3 (Two-way ANOVA

*for cell line P < 0.001 and time course, P < 0.001). The highest protein concentration was detected after 6 hr H_2O_2 exposure by densitometric immunoblot analysis. (*P < 0.05, **P<0.01, vs. corresponding control). Data are means ± SEM from 3 independent experiments.*

3.3 EFFECTS OF CASPASE INHIBITORS ON OXIDATIVE STRESS INDUCED CELL DEATH

3.3.1 Prevention of apoptosis by caspase inhibitors

Due to the enhanced activation of several caspases in response to oxidative stress the potential of several caspase inhibitors in protecting PC12 cells against oxidative stress-induced apoptosis was examined. In a pre-experiment, the caspase 3 inhibitor AC-DEVD-CMK was added in a concentration-dependent manner to APPsw transfected PC12 cells 3 hr or 1 hr prior to the exposure to 250 µM hydrogen peroxide for 24 hr. An experiment adding the caspase 3 inhibitor and hydrogen peroxide at the same time was also performed. As shown in Fig 3.13, best protection was provided adding caspases inhibitor at a concentration of 10 µM 3 hr prior hydrogen peroxide treatment.

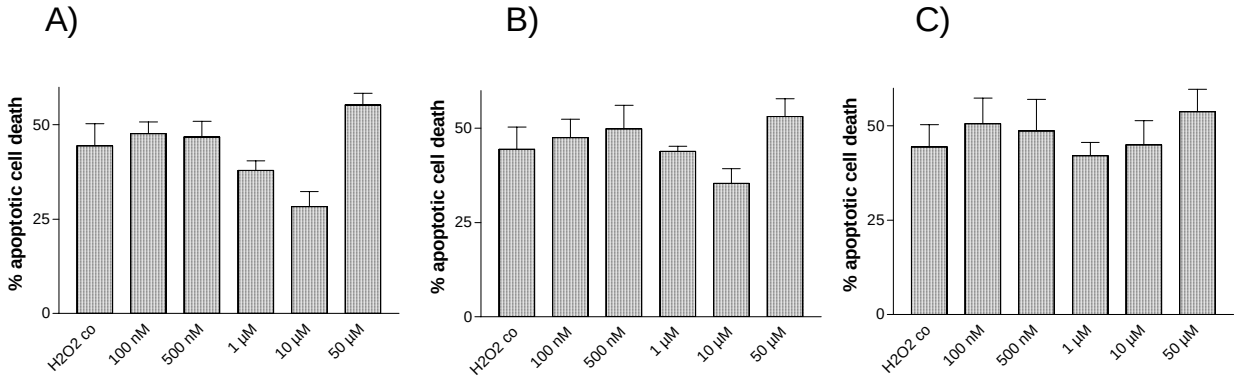

Fig. 3.13 Apoptotic cell death after treatment with AC-DEVD-CMK and H_2O_2 in APPsw transfected PC12 cells. Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. AC-DEVD-CMK was added to cells in a concentration dependent manner 3 hr (A) or 1 hr (B) prior H_2O_2, or at the same time as H_2O_2 (C). Best protection was measured, adding the caspase 3 inhibitor at a concentration of 10 µM 3 hr prior H_2O_2. Values are means ± SEM from 2-3 independent experiments.

Next, several PC12 cells were pre-treated with caspase inhibitors at a concentration of 10 µM 3 hr prior exposure to hydrogen peroxide to test their potential to inhibit oxidative stress-induced apoptosis. In addition a caspase inhibitor mix containing caspase 3, caspase 8 and caspase 9 (10 µM each) was also tested. As shown in Figure 3.14, only the inhibitor of the effector caspase 3, was able to reduce apoptotic rates in APPsw cells significantly. The pre-treatment of the cells with inhibitors of initiator caspase 8 and caspase 9 or the caspase inhibitor mix revealed only a tendency in preventing cells from apoptosis after a 24 hr hydrogen peroxide [250 µM] incubation. In a later experimental setting when the caspase 2 and caspase 6 inhibitor becomes available, they were also tested in preventing apoptosis. Here again, apoptosis could not be significantly avoided.

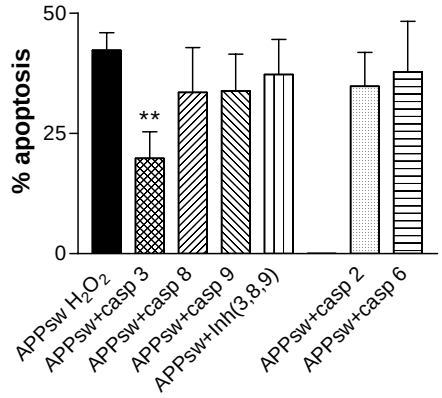

*Fig. 3.14 Apoptotic cell death of APPsw transfected PC12 cells. Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. Caspase inhibitors were added to cells 3 hr prior H_2O_2 exposure. The caspase 3 inhibitor strongly reduced apoptotic cell death in APPsw cells (**P < 0.01 student t-test). Values are means ± SEM from 6-8 independent experiments.*

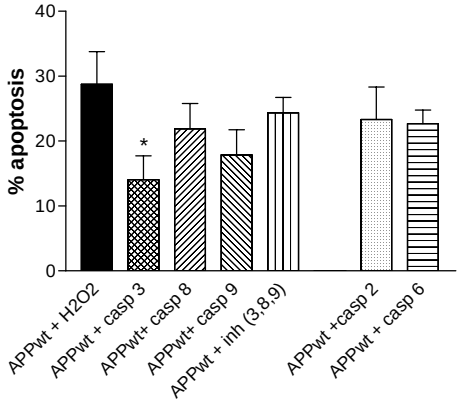

*Fig. 3.15 Apoptotic cell death of APPwt transfected PC12 cells, pre -treated with caspase inhibitors (*P < 0.05 student's t-test). Values are means ± SEM from 4-7 independent experiments.*

The obtained results using caspase inhibitors were similar for APPwt transfected PC 12 cells as APPsw. Significant protection was measured by a pre-treatment of the APPwt cells with 10 µM caspase 3 inhibitor 3 hr prior the addition of hydrogen peroxide. Here in particular, the caspase 9 inhibitor shows a pronounced tendency to prevent apoptosis in oxidative stress induced cell death. In vector transfected cells no significant protection could be observed using caspase inhibitors.

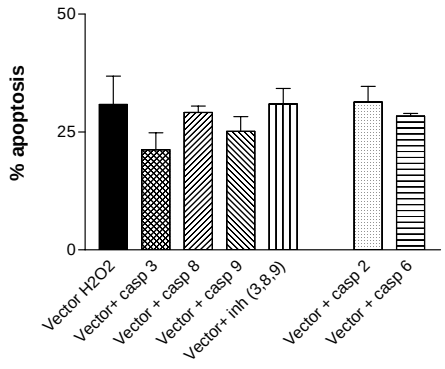

Fig. 3.16 Apoptotic cell death of empty vector transfected PC12 cells, pre-treated with caspase inhibitors. Values are means ± SEM from 4-6 independent experiments.

3.3.2 Reduction of caspase activity

The experiments using caspase inhibitors show that only the caspase 3 inhibitor was able to significantly reduce oxidative stress-induced apoptosis in APPsw and APPwt cells below the level of control cells. The inhibition of caspase 8, caspase 9, caspase 2 or caspase 6 was less effective in preventing apoptosis. Therefore, another control experiments was performed, measuring the caspase activities of cells pre-treated with caspase inhibitors prior to the

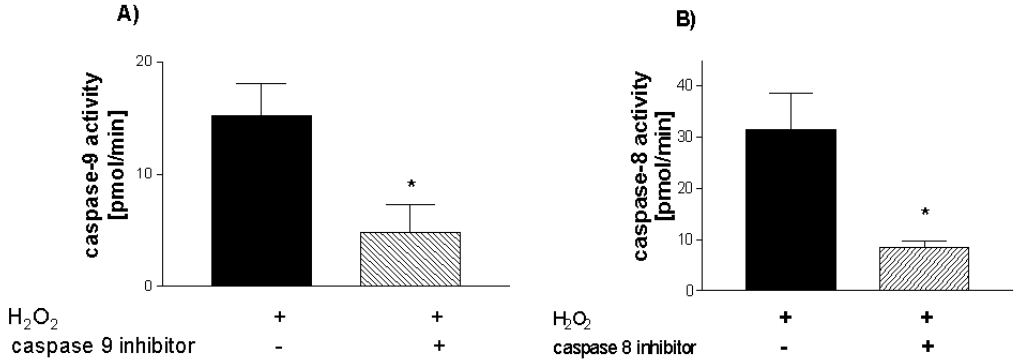

*Fig. 3.17 (A) Caspase 9 activity after 2 hr H_2O_2 incubation in cell lysates from APPsw-transfected PC12 cells without and with pre-treatment of 10 µM caspase 9 inhibitor. (B) Caspase 8 activity in cell lysates from APP-transfected cells after 2 hr of H_2O_2 induction (*P < 0.05, student's t-test). Data are means ± SEM from 3-7 independent experiments.*

treatment with hydrogen peroxide. As controls, caspase 8 and caspase 9 were measured, and in fact, reduced activities were detected. This means that although the treatment of PC12 cells with hydrogen peroxide leads to activation of these initiator caspases, their inhibition is not sufficient enough to avoid apoptosis. The lack of efficacy of the initial caspase inhibitors to abolish apoptotic cell death could be attributed to the further consisting compensatory pathway. In another experiment, caspase 3 activity was measured in lysates of cells pre-treated with caspase 8, caspase 9 or caspase 2 inhibitor. Here, a reduced caspase 3 activity could be detected in caspase 8 -and caspase 9 inhibitor pre-treated cells.

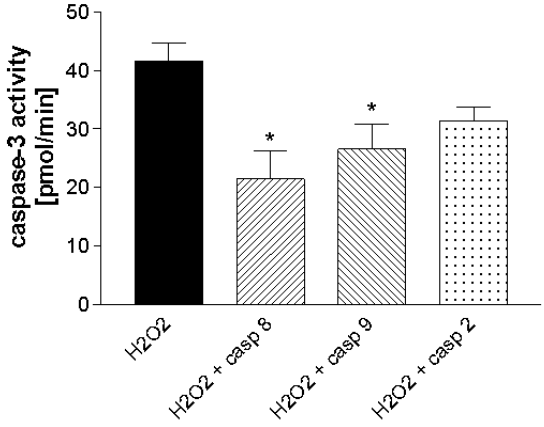

*Fig. 3.18 (A) Caspase 3 activity after 6 hr H_2O_2 incubation in cell lysates from APPsw-transfected cells. Pre-treatment of cells with 10 µM caspase 8, caspase 9 or caspase 2 inhibitor reduce caspase 3 activity significantly (*P < 0.05, paired t-test). Data are means ± SEM from 3-6 independent experiments.*

3.3.2 Necrotic cell death

The incubation of PC12 cells with increasing concentrations of hydrogen peroxide for 24 hr results in increasing apoptotic levels concentration-dependent manner, with the highest apoptotic levels using a concentration of 500 µM hydrogen peroxide (Eckert 2001, Fig. 3.19). Higher concentrations results in a decrease of apoptotic cells. Therefore, experiments were performed using trypan blue dye, which penetrates into dead cells due to a disturbed cell membrane, while vital cells are undyed. Since plasma membrane leakage is also a feature of necrotic cell death, both apoptotic and necrotic cells are dyed and can be distinguish from vital cells. Using increasing concentrations of hydrogen peroxide, the number of death cells increases in a concentration-dependent manner reaching approximately 100% at a concentration of 1000 µM hydrogen peroxide. This shows that concentrations higher than 250

µM hydrogen peroxide results in an increase in the number of necrotic cells, which may explain the decrease of apoptotic levels upper 500 µM hydrogen peroxide.

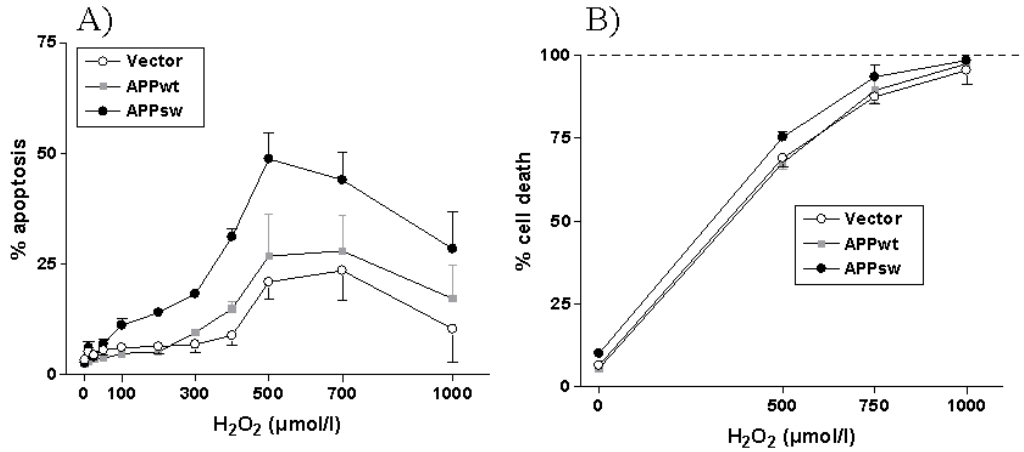

Fig. 3.19 (A) Increase of apoptotic cells after treatment with increasing concentrations of H_2O_2. (B) Increase of trypan blue dyed cells after 24 hr incubation of increasing concentrations of H_2O_2. Values are means ± SEM from 3-4 independent experiments per treatment.

In addition, cell death of 500 µM hydrogen peroxide incubated cells pre-treated with caspase inhibitors were determinated using trypan blue. Here, no caspase inhibitor was able to reduce absolute cell death significantly, neither in APPsw, APPwt nor empty vector transfected PC12 cells.

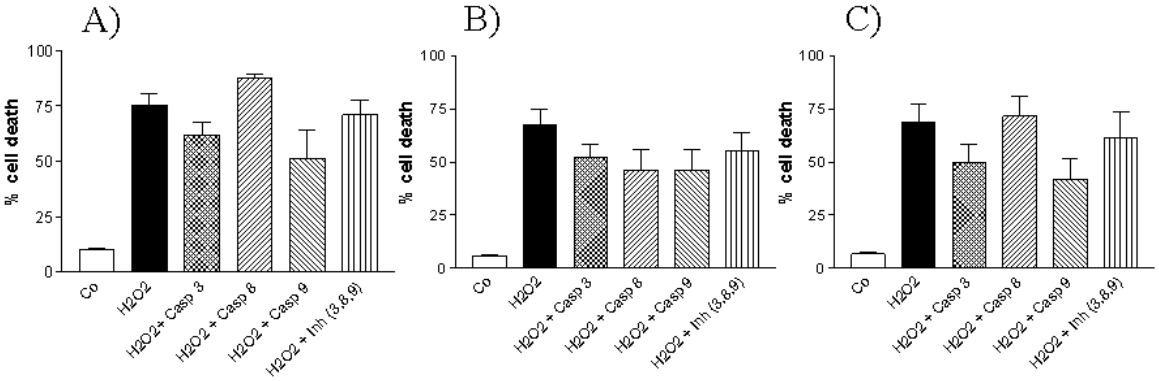

Fig. 3.20 Cell death levels of APPsw transfected PC12 cells. (A) APPwt transfected PC12 cells (B) and vector transfected cells (C). Cell death was assessed by trypan blue dye. Caspase inhibitors were added to cells in 3 hr prior H_2O_2 exposure. Values are means ± SEM from 6-9 independent experiments.

3.3.3 Protection of metabolic activity by caspase inhibitors

In addition to propidium iodide and trypan blue staining, the MTT assay was performed to test, whether caspase inhibitors show protective effects on metabolic activity in oxidative stress- induced cell death. In a pre-experiment the conversion of the MTT reagent by mitochondrial oxidation-reduction reaction to formazan crystals were measured in APPsw, APPwt and empty vector transfected PC12 cells. Noticeable was the reduced metabolic activity in APPsw PC12 cells compared to APPwt and to vector transfected cells under basal culture conditions (Fig. 3.21A). An incubation for 24 hr with increasing concentrations of hydrogen peroxide reduces metabolic activity as shown in Fig. 3.21B.

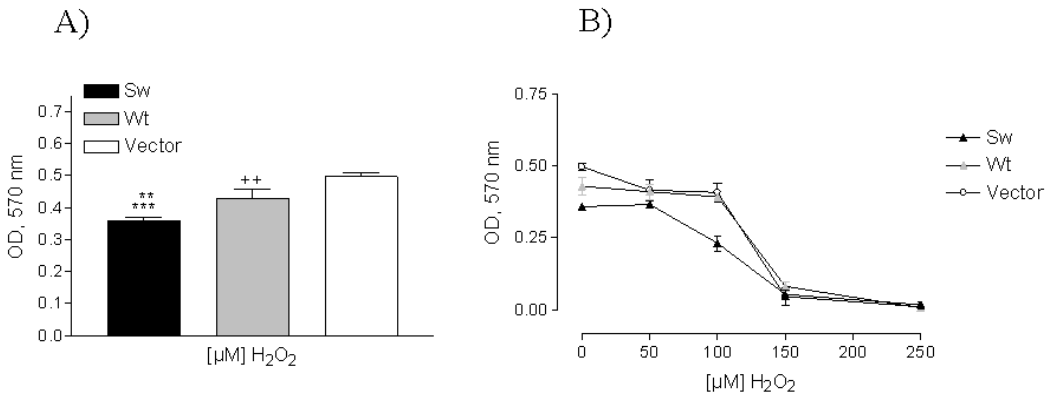

*Fig. 3.21 (A) Metabolic activity of PC12 cells (***P < 0.001, ++P < 0,01, student t-test vs. vector, **P < 0,01, vs. Wt). (B) H_2O_2-induced decrease of metabolic activity in a concentration dependent manner. Values are means ± SEM from 3 independent experiments.*

Caspase inhibitors (10 µM) were added 3 hr before the incubation of PC12 cells with different concentrations of hydrogen peroxide. Only using 100 µM hydrogen peroxide, a protective effects was measured using caspase 3 inhibitor in vector transfected cells (Fig. 3.22C).

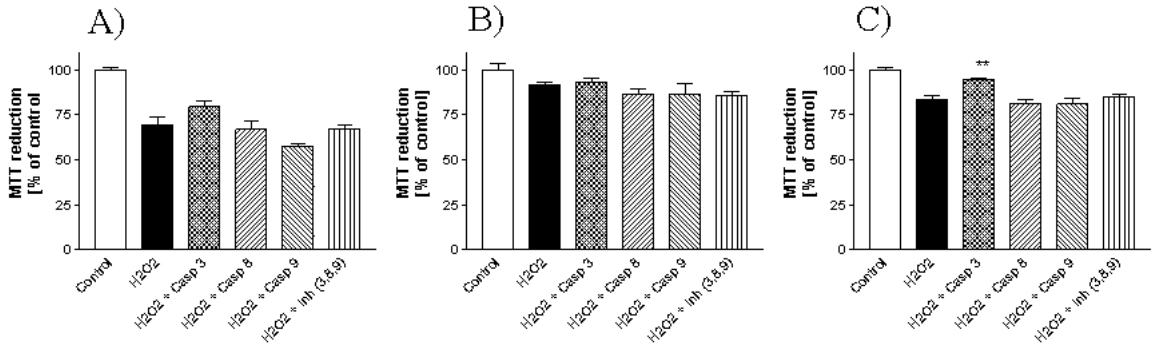

Fig. 3.22 (A) Metabolic activity of PC12 cells incubated for 24 hr with 100 µM H_2O_2. (A)

*APPsw, (B) APPwt and (C) vector (**P < 0.01, studend's t-test vs. H_2O_2). Values are means ± SEM from 3 independent experiments.*

Incubating PC12 cells with 150 µM hydrogen peroxide, a protective effect was found for caspase 3 and caspase 8 inhibitor, again only in vector transfected cells (Fig. 3.23C).

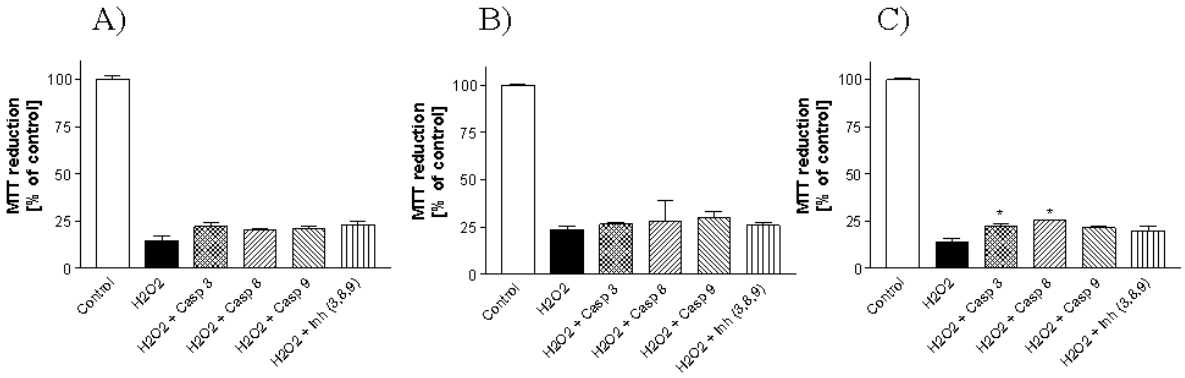

*Fig. 3.23 (A) Metabolic activity of PC12 cells incubated for 24 hr with 150 µM H_2O_2. (A) APPsw, (B) APPwt and (C) vector (*P < 0.01, student t-test vs. H_2O_2). Values are means ± SEM from 3 independent experiments.*

At a concentration of 250 µM hydrogen peroxide, a significant protection could be detected with all used caspase inhibitors (caspase 3, caspase 8, caspase 9, caspase inhibitor mix) for APPsw and APPwt as well as for vector transfected cells. Thus, caspase inhibitors show best protection inducing oxidative stress with 250 µM hydrogen peroxide, in which also the highest caspase activities have been measured (Eckert 2001). The different results for protective effects of caspase inhibitors measuring apoptosis with propidium iodide staining, membrane, membrane leakage with trypan blue dye and metabolic activity with the MTT assay may not be surprising. The inhibition of caspases may finally result in a maintain of several metabolic pathways. Nevertheless, cells die by apoptosis or necrosis, because the metabolic activity is still not sufficient (about 25 % of basal controls) for survival.

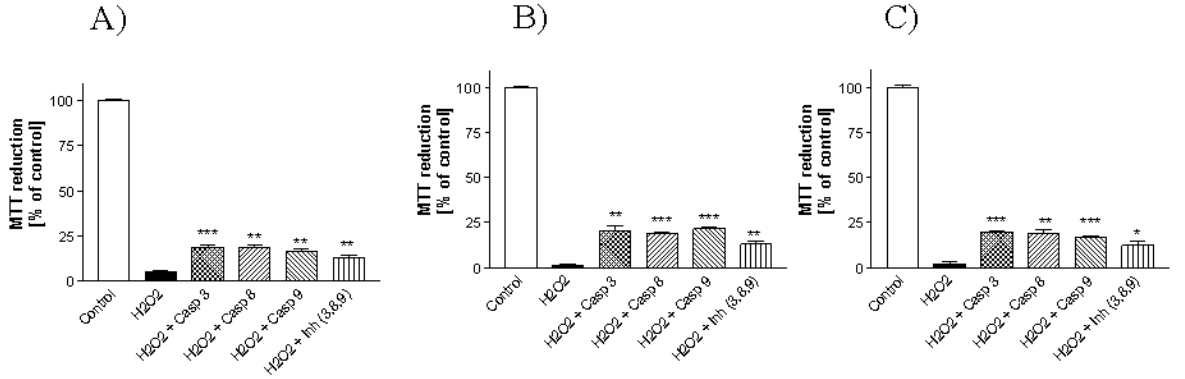

*Fig. 3.24 (A) Metabolic activity of PC12 cells incubated for 24 hr with 250 µM H_2O_2. (A) APPsw, (B) APPwt and (C) vector (***P < 0.001, **P < 0.01, **P < 0.05 student t-test vs. H_2O_2). Values are means ± SEM from 3 independent experiments.*

3.3.4 Protection of mitochondrial membrane potential by caspase inhibitors

Interestingly, recent data describe caspase 2 to be localized in several intracellular compartments, including the mitochondria, Golgi, cytosol, and nucleus (Mancini 2000, Lassus 2002, Paroni 2002). Cytotoxic stress causes activation of caspase 2, which is required for the permeabilization of mitochondria (Lassus 2002) and induces the release of cytochrome C (Robertson 2002).

Since mitochondria play a vital role in energy metabolism, and mitochondrial dysfunction has been identified in a large proportion of neurodegenerative disease including AD (Eckert 2003) it was investigated, whether the pre-treatment of PC12 cells with caspase 2 inhibitor prior to the induction of oxidative stress protects mitochondrial functions. Therefore, oxidative stress was induced for 6 or 24 hr, and the mitochondrial membrane potential measured using the structural marker for mitochondria and the indicator of mitochondrial membrane potential Rhodamine 123. Caspase 2 inhibitor was added at 4 µM and 10 µM before the exposure of cells to 250 µM or 500 µM hydrogen peroxide.

Here, the results varied depending on time points of oxidative stress induction or caspase 2 inhibitor concentration. After an induction for 24 hr with 250 µM hydrogen peroxide a significant protection was observed with 4 µM caspase 2 inhibitor in APPsw PC12 cells, while in APPwt PC12 cells only a using 4 µM caspase 2 inhibitor was measured. In vector controls protection was detected using 10 µM caspase 2 inhibitor.

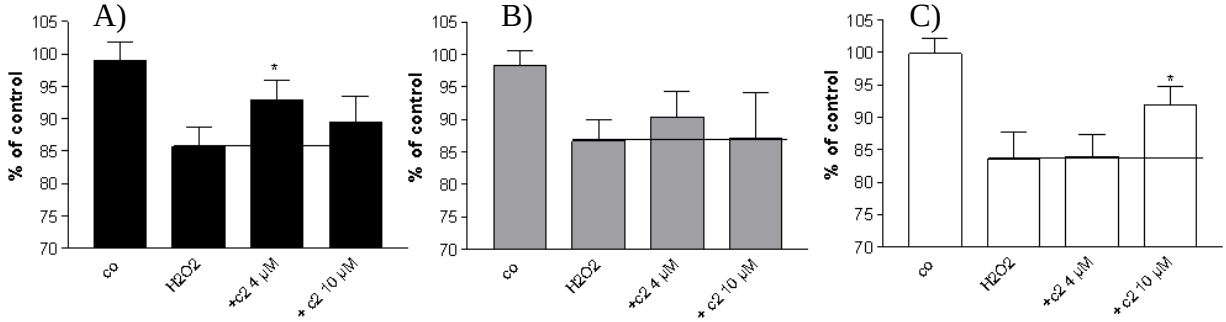

Fig. 3.25 Mitochondrial membrane potential analysis: An incubation with 250 µM H_2O_2 for 24 hr leads to a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) in PC12 cells. (A) APPsw, the pre-treatment with 4 µM caspase 2 inhibitor protects the mitochondria against oxidative damage. (B) APPwt. (C) In vector transfected PC12 cells the pre-treatment with 10

*µM caspase 2 inhibitor protects mitochondria. (*P <0.01 vs. corresponding H_2O_2-treated cells, paired t-test). Values are means ± SEM from 6-10 independent experiments.*

Using 500 µM hydrogen peroxide a significant protection was measured after an incubation of 6 hr and pre-incubating APPsw PC12 cells with 10 µM caspase 2 inhibitor (Fig 3.26). Here neither in APPwt nor in vector controls significant protection of caspase 2 inhibitor could be detected, confirming the substantial role of caspase 2 in cell death of APPsw cells.

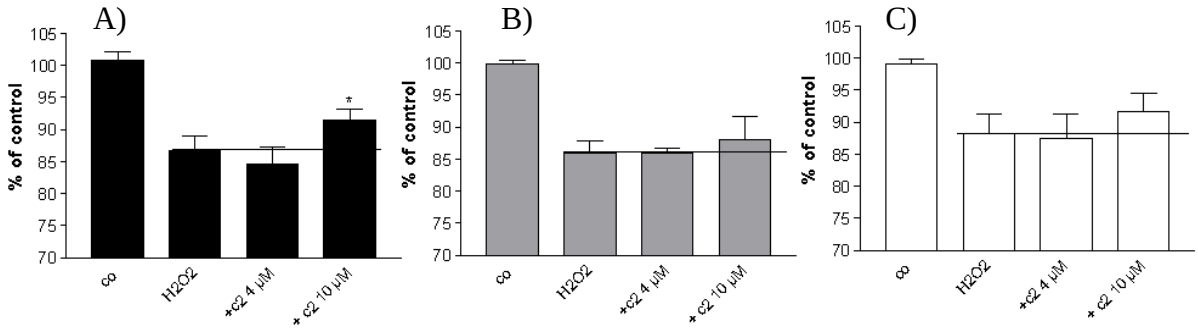

*Fig. 3.26 Mitochondrial membrane potential of PC12 cells after an incubation with 500 µM H_2O_2 for 6 hr. (A) APPsw, the pre-treatment with 10 µM caspase 2 inhibitor protects the mitochondria against oxidative damage. (B) APPwt. (C) vector. (*P <0.01 vs. corresponding H_2O_2-treated cells, paired t-test). Values are means ± SEM from 6-10 independent experiments.*

3.4 ACTIVATION OF JNK DURING OXIDATIVE STRESS IN PC 12 CELLS

3.4.1 Aß doses effect of APP on JNK activation

The mobilization of the stress-activated protein kinase (JNK) plays an important role in apoptosis induced by several types of environmental stress, like UV, γ-radiation, inflammatory cytokines, and DNA damage (Sanchez 1994, Mesner 1995, Ghahremani 2002). In oxidative stress-induced cell death, the activation of mitochondrial apoptosis machinery by JNK in adult cardiac myocytes has been observed (Aoki 2002).

Hence, the question whether oxidative stress-mediated activation of JNK pathway is affected by overexpression of APP was addressed. Western blotting of immunocomplexes to detect activation of JNK and c-Jun was performed. Hydrogen peroxide treatment of APPsw PC12 cells induced JNK phosphorylation within 2 hr (Fig. 3.27A). The highest expression was detected after 4 hr with an elevated expression in APPsw cells compared to APPwt cells and vector controls. When activated, JNK translocates to the nucleus to regulate transcription through its effects on c-Jun and other transcription factors. As for JNK we observed a strong impact of the Swedish APP transfection on the activation of c-Jun (Fig. 3.27B). In these experiments, the kinetics of activation of c-Jun correlated with the phosphorylation of JNK with the highest detection of phoshorylated c-Jun after 6 hr of H_2O_2 treatment. The expression of phosphorylated c-Jun was markedly elevated in APPsw compared to treated APPwt or vector transfected controls after 4 and 6 hr hydrogen peroxide treatment.

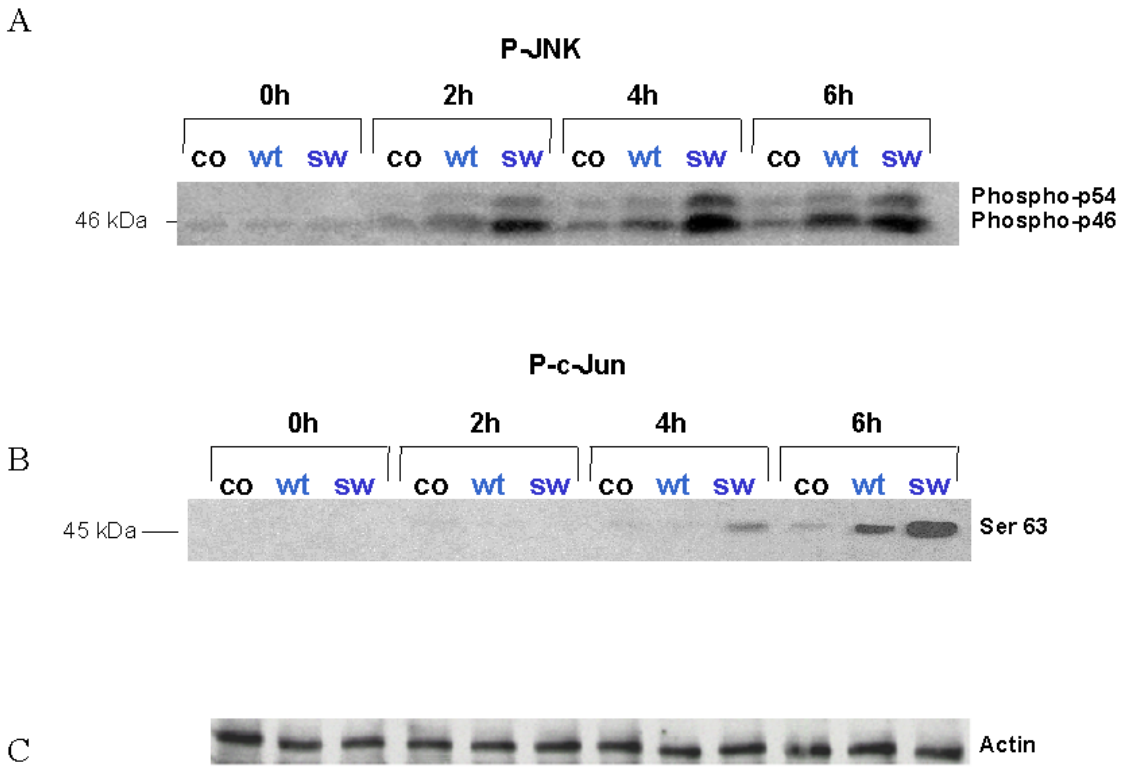

Fig. 3.27 Activation of JNK and c-Jun in oxidative stress-induced cell death in APPsw, APPwt and vector-transfected cells analysed by Western blotting using anti-active p-JNK and p-c-Jun antibodies for the analysis of SAPK/JNK and c-Jun phosphorylation/activity. (A) APPsw cells exhibit significantly enhanced protein expression levels of JNK activation in PC12 cells compared to APPwt and vector transfected cells. Maximal activated p-JNK was detected after 4 hr exposure to oxidative stress. (C) The expression of phosphorylated c-Jun was markedly elevated in APPsw cells compared to H_2O_2-treated APPwt and vector controls after

hydrogen peroxide treatment. Maximal amount of p-c-Jun was detected after 6 hr H_2O_2 treatment. (C) The blots were reprobed with an anti-actin antibody to confirm the equal loadings of the protein amounts.Data are from a representative experiment repeated at least three times with similar findings.

3.5 EFFECTS OF JNK INHIBITOR SP600125 ON OXIDATIVE STRESS INDUCED CELL DEATH

3.5.1 Effects on caspase activity

As detected by western blotting, the transfection of the Swedish FAD mutation in PC 12 cells leads to an enhanced activation of the JNK pathway under oxidative stress. Therefore, the effect of SP600125, a selective anthrapyrazolone inhibitor of JNK (Bennet 2001), was investigated. The activity of different caspases was measured in PC12 cells pre-treated with JNK inhibitor to attribute the JNK involvement in oxidative stress-induced apoptosis. A highly significant reduction of caspase 9 activity (Two-way ANOVA $P < 0.001$) and caspase 3 activity (Two-way ANOVA $P = 0.0384$) in cell lysates of APPsw cells pretreated with 200 nM JNK inhibitor was found, whereas the activities of caspase 2 and caspase 8 were not significantly altered (Fig. 3.28).

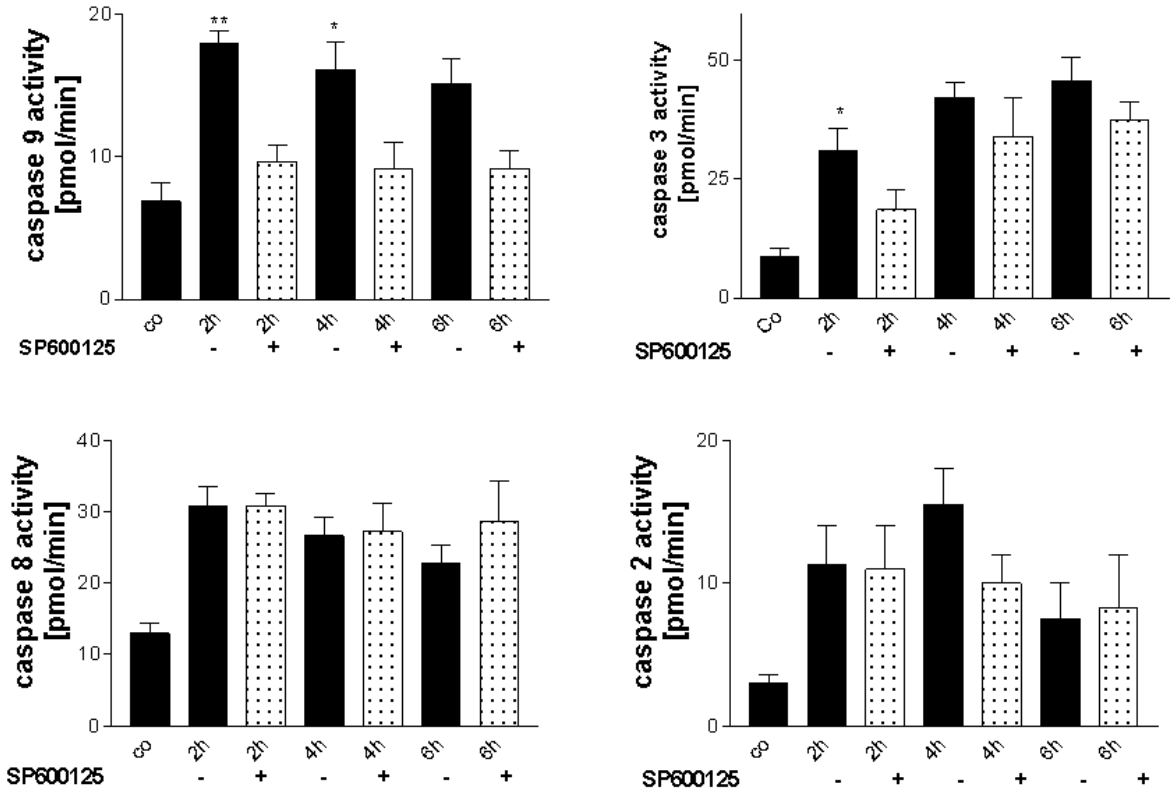

*Fig. 3.28 Reduction of caspase activities by JNK-inhibitor SP60125: Cells were pretreated with 200 nM SP 600125 and incubated with H_2O_2 in a time dependent manner. Caspase activities were measured in cytosolic protein extracts. Caspase 9 activity in cell lysates of APPsw cells pretreated with JNK inhibitor was significantly reduced compared to APPsw cells without pretreatment with the inhibitor (two-way ANOVA: p< 0.001 for pretreatment). The maximal reduction of caspase 9 activity by pretreatment of the cells with JNK inhibitor was measured after 2 hr exposure to oxidative stress (**P < 0.01 vs. corresponding pretreated cells, *P < 0.05 vs. corresponding pretreated cells after 4hr H_2O_2 induction). Caspase 3 was also reduced by the JNK inhibitor (Two-way ANOVA: P < 0.01 for time course and pretreatment) with maximal reduction of caspase 3 activity after 2 hr exposure to oxidative stress (*P < 0.05 vs. corresponding pretreated cells). The activity of caspase 2 and caspase 8 were not significantly altered. Values are means ± SEM from 4-6 independent experiments.*

3.5.2 Protection of mitochondria

Since a reduction of caspase 9 was observed using SP600125 (see. 3.5.1), we measured if the mitochondria are protected from the collapse of the membrane potential, which normally occurs when oxidative stress is induced (Keil 2004). Corresponding to the reduction of

caspase 9 activity, we were able to detect a protection against a decrease in the mitochondrial membrane potential by SP600125 (Fig. 3.29) after the induction of oxidative stress for 6 hr, indicating an involvement of JNK upstream of mitochondria in oxidative stress signalling.

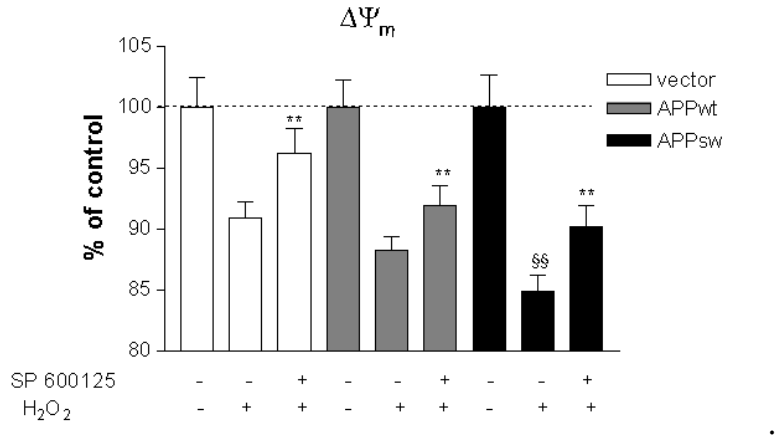

*Fig. 3.29 Mitochondrial membrane potential analysis: oxidative stress leads to a decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) in PC12 cells. The strongest reduction of the mitochondrial membrane potential occurs in APPsw cells ($^{\$\$}P$ <0.01 vs. corresponding H_2O_2-treated vector cells, t-test). The pre-treatment of cells with SP600125 before exposure to H_2O_2 significantly protects the mitochondria against oxidative damage and decrease in $\Delta\Psi_m$ in all three cell lines (**P <0.01 vs. corresponding H_2O_2-treated cells, paired t-test).*

3.5.3 JNK activation in mitochondrial fraction

Since JNK inhibition shows protective effects on mitochondrial membrane potential when oxidative tress is induced, mitochondrial fractions were isolated from PC12 cells, and examined whether JNK translocates into mitochondria during oxidative stress. While nearly no activated JNK was found in untreated PC12 cells, after 2 hr of incubation with 250 µM hydrogen peroxide, slight JNK activation could be detected. Of note, in PC12 cells containing the Swedish APP mutation, activated JNK was also found in mitochondrial fraction after 4 and 6 hr induction.

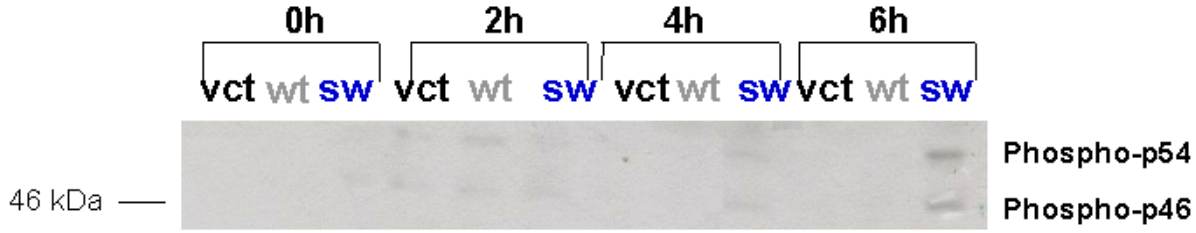

Fig. 3.30 mitochondrial translocation of JNK during oxidative stress in PC12 cells. Data are from a representative experiment repeated at least three times with similar findings.

3.5.4 JNK inhibitor studies on apoptosis

As shown in 3.52 the JNK inhibitor SP600125 protects mitochondria against a decrease in the mitochondrial membrane potential which occurs when oxidative stress is induced. Also a reduction in the caspase 9 and caspase 3 activity in cell lysates of APPsw cells pre-treated with 200 nM JNK inhibitor was observed. In addition, it was investigated whether the JNK inhibitor reduces apoptotic levels of APPsw PC12 cells after 24 hr of hydrogen peroxide (250 µM) incubation. The treatment with hydrogen peroxide led to a significant increase of apoptotic cells in APPsw-bearing PC12 cells (42.21% ±3.71%) compared to vector cell line (30.87% ± 5.97%), APPsw cells pre-treated with the 200 nM JNK inhibitor (33.77 % ± 4.06%) exhibited similar apoptotic cell levels as the corresponding vector cells (Fig 3.31A). Higher concentrations of JNK inhibitor were less protective in preventing oxidative stress induced apoptosis, because at high concentration the JNK-inhibitor is toxic itself (Fig. 3.31B). In PC12 cells cultured under basal conditions, without other stimulation, it was found that 10,000 nM JNK inhibitor enhanced apoptotic levels significantly.

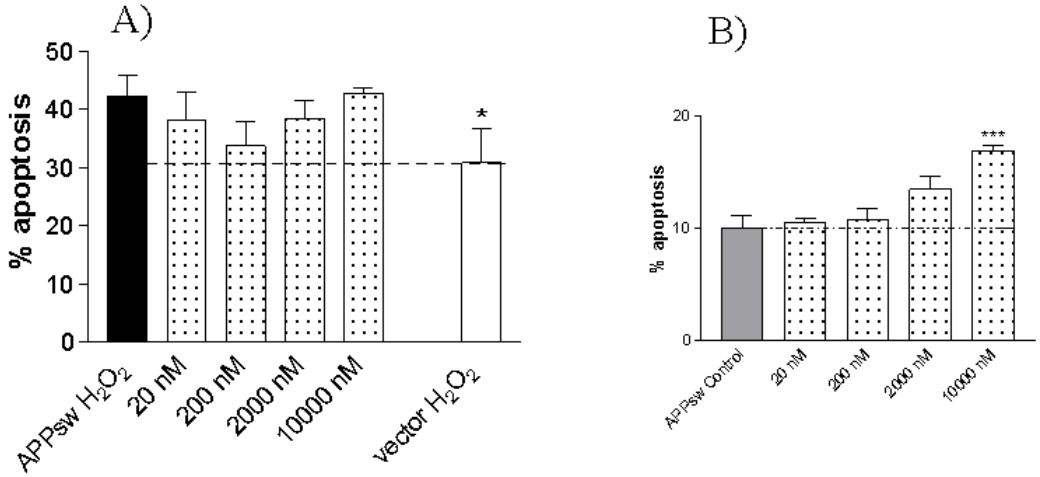

Fig. 3.31 Apoptotic cell death after treatment with H_2O_2 in PC12 cells. (A) APPsw PC12 cells showed enhanced sensitivity to oxidative stress-induced death compared to vector cells ($^{+}P < 0.05$, student's t-test). In parallel experiments cells were pretreated for 3 hours with different concentrations of the JNK inhibitor SP600125) and then exposed to H_2O_2 for 24 hr. Pre-treatment of APPsw cells with JNK inhibitor at a concentration of 200 µM reduced

apoptotic cell death to levels corresponding to that of vector cells. Values are means ± SEM from 6-8 independent experiments. (B) High concentrations of JNK inhibitor (10 000 nM) enhance basal apoptotic levels of APPsw PC12 cells ($^{***}P < 0.001$, student's t-test, vs. APPsw control). Values are means ± SEM from 5-6 independent experiments.

3.6 MESSENGER RNA EXPRESSION OF APOPTOTIC FACTORS DURING OXIDATIVE STRESS IN PC12 CELLS OVEREXPRESSING APP

3.6.1 Members of the Bcl-2 family

Since the mitochondrial apoptotic pathway is regulated by proteins of the Bcl-2 superfamily, the expression of the pro-apoptotic Bax- and the antiapoptotic Bcl-2 proteins were investigated in oxidative stress induced cell death in PC12 cells. After an incubation of the indicated periods of time with 250 µM hydrogen peroxide, total RNA was isolated, transcript into a cDNA bank, and a PCR using appropriated designed primer (see. 2.3.6) was run. For Bax, nearly no increase in the mRNA was found after induction of hydrogen peroxide induced cell death in PC12 cells. Here the expression was also not altered between APPsw-, APPwt- or vector transfected PC12 cells. Similar results were detected by western blotting concerning the protein expression by Astrid Bonert from our group in parallel experiments (Keil 2004).

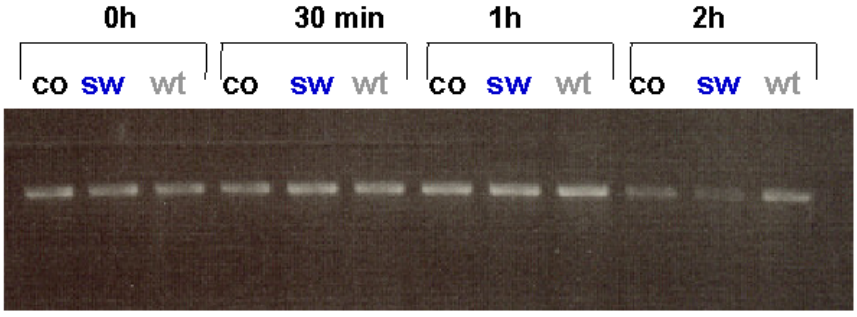

Fig. 3.32. Expression of Bax mRNA during oxidative stress in PC12 cells Data are from a representative experiment repeated at least three times with similar findings.

Analysing the expression results of Bcl-2 in PC12 cells was surprising, because no Bcl-2 mRNA expression could be detected using appropriate and verified primer for rat Bcl-2. After checking the literature, in fact, a publication was found, showing also no Bcl-2 protein

expression in PC 12 cells (Maroto 1997). This was confirmed by Astrid Bonert from our lab, perfoming western blotting.

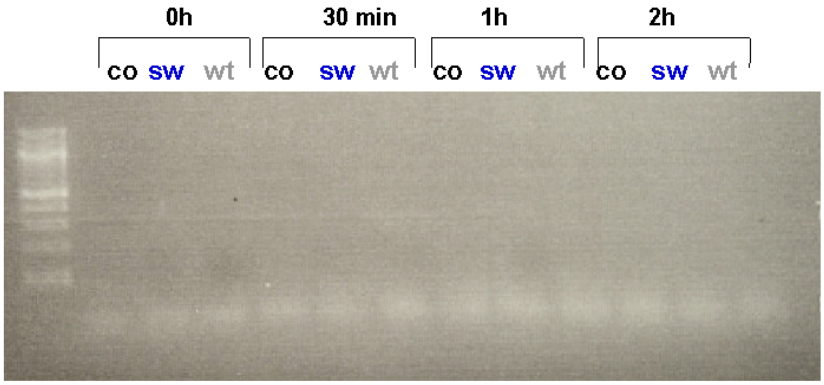

Fig. 3.33. PC12 cells show no BCL-2 mRNA expression, Data are from a representative experiment repeated at least three times with similar findings.

3.6.2 Apoptosis inducing factor

AIF is a recently discovered flavoprotein that resides in the mitochondria and identified as a cell death molecule (Susin 1999). Upon death stimuli, AIF translocates from the mitochondria to the nuclei and induces large-scale DNA fragmentation (> 50 kbp) and cell death in a caspase-independent manner (Daugas 2000). Here, the RT-PCR analysis show an oxidative stress induced increase in the expression of mRNA in PC12 cells, whereas the expression was higher in both, APPsw and APPwt cells compared to vector controls after 1 hr hydrogen peroxide induction. An enhanced AIF protein expression was also found by Astrid Bonert in mitochondrial fractions of PC12 cells.

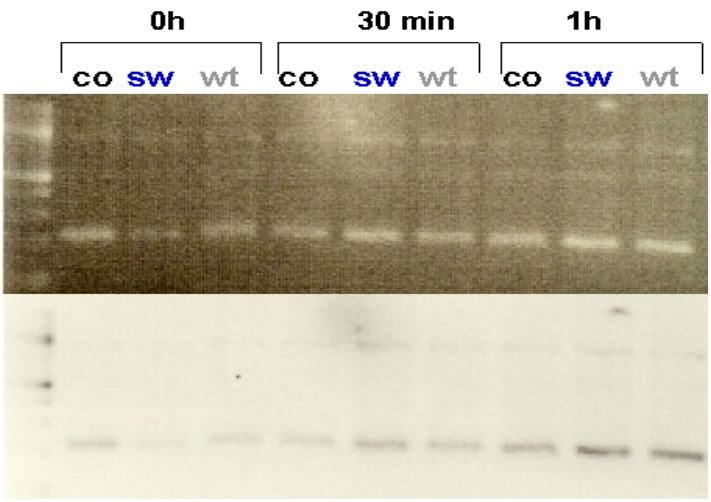

Fig. 3.34. Expression of AIF mRNA increases during oxidative stress in PC12 cells. Data are from a representative experiment repeated at least three times with similar findings.

3.6.3 Glycogen synthesis kinase 3ß

Glycogen synthase kinase 3ß (GSK3ß) was initially described as a key enzyme involved in glycogen metabolism, phosphorylating and inactivated glycogen synthase the final enzyme in glycogen biosynthesis. However it is known to regulate many cell functions. GSK3ß is a component of the WNT signalling pathway with a role in cell proliferation, differentiation, adhesion, microtubule dynamics, apoptosis, and fast axonal transport (Frame 2001, Morfini 2002). GSK3ß, a serine/threonine kinase, is highly expressed in brain tissue (Mandelkow 1999) and has been linked to primary abnormalities associated with Alzheimer's disease. These include participation of GSK-3 in phosphorylating the microtubule binding protein tau that may contribute to the formation of neurofibrillary tangles (Mandelkow 1999, Sang 2000) and interactions of GSK-3ß with presenilin (Kirschenbaum, Morfini 2002b) or AICD (Kim 2003).

A very recent online published report shows that the c-terminal fragment of APP (AICD) exert neurotoxicity on PC 12 cells and rat primary cortical neurons by inducing the expression of GSK3ß, and the Swedish mutant form of APP770 induced the up-regulation of GSK3ß at both the mRNA and the protein levels (Kim 2003). In another recent publication a elevated endogenous GSK3ß kinase activity in HEK cells expressing the Swedish APP751 mutation has been described (Ryder 2004). Therefore the mRNA levels in APP-transfected PC12 cells were analysed using appropriate primers.

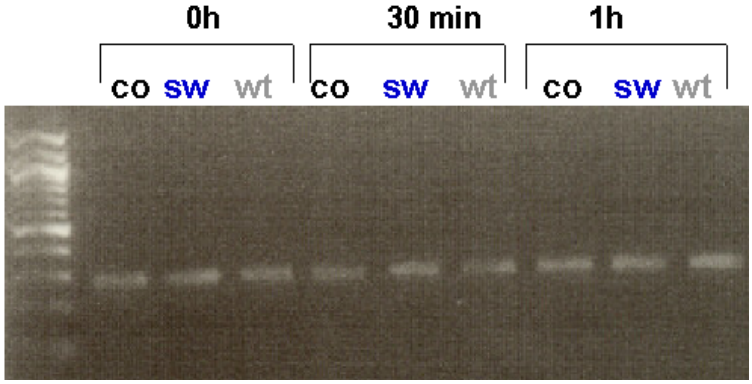

Fig. 3.35 Expression of GSK3ß mRNA is unaltered between APPsw, APPwt and vector-transfected PC12 cells. Data are from a representative experiment.

In contrast to Kim et al (2003), concerning to the GSK3 mRNA expression no effects of the Swedish mutation were detected in PC12 cells. Also no differences in mRNA expression of GSK3ß were found in vector-transfected control cells. Since GSK3ß is regulated by inhibition through phosphorylation of Ser9 by protein kinase B/Akt (Frame 2001b) the activation of GSK3ß may be rather trough down regulation of the protein kinase B than through altered mRNA expression.

3.6.4 GAPDH

As control, RT-PCR from the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was performed from the same cDNA bank. As shown in Fig. 3.31 the GAPDH mRNA expression was unaltered.

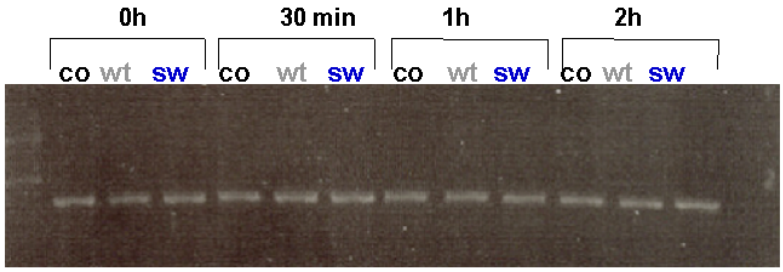

Fig. 3.36. GAPDH mRNA expression in PC12 cells. Data are from a representative experiment repeated at least three times with similar findings.

3.7 REDUCTION OF TROPHIC SUPPORT ENHANCE APOPTOSIS IN APPSW EXPRESSING PC12 CELLS

3.7.1 DNA ladder

Cells require serum to maintain growth in vitro. Serum provides growth and survival factors and its removal causes cellular stress. In parallel experiments APP- transfected PC12 cells has also been analysed by Dr. Steffen Leutz for their susceptibility to cell death induced by the reduction of trophic support or by additional treatment with staurosporine (Leutz 2002). The expression of APPsw markedly enhanced the level of apoptotic PC12 cells induced by serum reduction. Likewise, the expression of APPsw rendered PC12 cells more vulnerable to staurosporine but only under serum-reduced conditions. One feature of apoptosis is that chromosomal DNA is enzymatically cleaved to 180-bp internucleosomal fragments. Therefore, to confirm the apoptotic nature of cell death after trophic factor withdrawal, genomic DNA was isolated and DNA laddering was assessed in APP-expressing PC12 cells grown under serum-reduced conditions as shown in Fig. 3.37. While APP-expressing PC12 cells grown in regular culture medium with 15% serum have a complete genomic DNA, the APP-expressing PC12 cells grown for 24 hr in serum free medium contain fragmented genomic DNA.

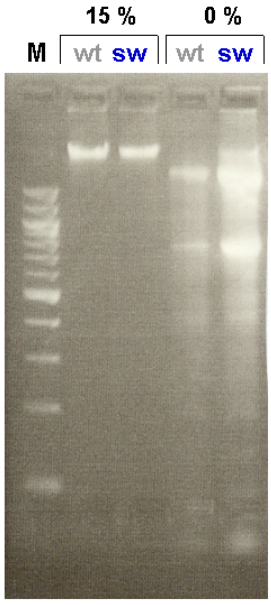

Fig. 3.37. Reduction of serum concentration induces apoptosis as indicated by typical DNA laddering. APPwt- and APPsw-expressing Pc12 cells exhibit ladder-like DNA fragmentation after withdrawal of trophic factors for 24 hr. No DNA laddering is detected when cells were grown in regular culture medium (15%).

3.8 A CELL MODEL REFLECTING HIGH Aß LOAD

3.8.1 Characterization of HEK cell lines overexpressing APPwt and APPsw

As an additional cell system to PC12 cells, HEK (human embryonic kidney 293) cells were also stably transfected with APPwt or APPsw cDNA. HEK cells are non neuronal cells but are often used in transfection experiments in laboratories because of the high transfections rates. The characterisation of HEK cells overexpressing APPwt or APPsw has been performed by Astrid Bonert from our lab showing similar results concerning the APP expression. The Aß(1-40) production of HEK cells transfected with APPwt or APPsw is shown in Fig. 3.38. Culture supernatants of the APPsw transfected HEK cells showed elevated Aß(1-40) levels compared to HEK cells overexpressing APPwt, whereas the differences in the Aß production were more pronounced as in PC12 cells (Fig. 3.1). HEK APPsw cells showed a tenfold increase in the Aß(1-40) production compared to HEK APPwt cells. Of note, HEK cells bearing the APPsw mutation exhibit a 30-fold increased Aß production compared to APPsw bearing PC12 cells (Fig. 3.1).

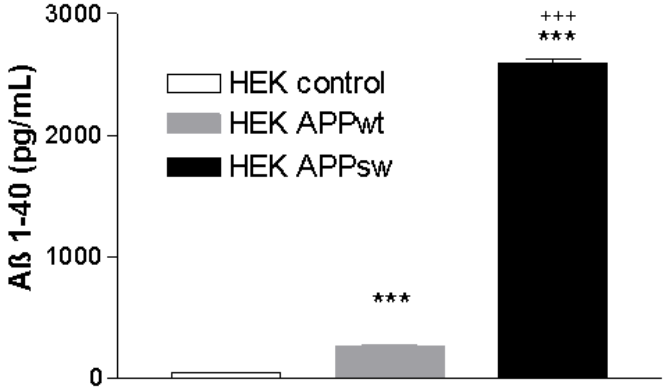

*Figure 3.38 Conditioned media analysed for Aß in HEK cells: HEK cells expressing the Swedish mutant APPsw showed a tenfold increase in the Aß(1– 40) production when compared to the HEK APPwt cells (ANOVA ***P<0.001: $^{+++}$P< 0.01 vs. APPwt, ***P<0.001vs. control, posthoc Tukey's). The increase in the production of Aß(1– 40) was confirmed in three independent experiments. HEK cells bearing human APPwt showed a nine fold increase in Aß production compared to controls (***P<0.001 vs. control, posthoc Tukey's). Values are means ± SEM from three independent experiments*

3.8.2 Basal apoptosis is already increased in APPsw HEK cells

Apoptosis in HEK cell populations was measured by propidium iodide staining and flow cytometry anaylsis. In contrast to APPsw PC12 cells, which show no increased basal apoptosis (Eckert 2001), the APPsw-transfection of HEK cells leads to increased levels of apoptotic cells under basal conditions. Thus, APPsw PC12 cells seems to be able to compensate the consequences of the increased Aß levels, while the 30 fold higher Aß levels in APPsw HEK cells compared to APPsw PC12 cells, lead directly to increased levels of apoptotic cells even under basel conditions (Fig. 3.39). Additionally, treatment of PC12 cells extracellularly with $Aß_{1-42}$ (100 nM) results also in increased apoptotic cell death (Keil 2004). Thus, under basal conditions APPsw PC12 cells are able to compensate the consequences of the increased Aß production. In contrast, APPsw HEK cells expressing high amounts of Aß, show already increased level of apoptotic cells under baseline conditions.

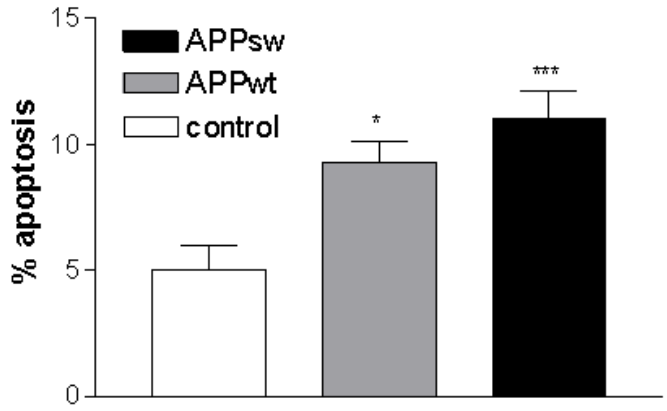

*Figure 3.39 Increase basal apoptotic levels in HEK cells overexpressing APPsw mutation compared to controls cells (ANOVA ***p<0.001: ***p<0.001 vs. control, posthoc Tukey's). Also HEKwt cells have enhanced basal apoptotic levels vs. controls (*p< 0,05, posthoc Tukey's). Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. Values are means ± SEM from eight independent experiments*

3.8.3 Oxidative stress induced cell death in HEK cells

Similar to PC12 cells, apoptosis was induced in HEK cells using the oxidative stressor hydrogen peroxide and the nuclear DNA fragmentation was quantitatively detected by propidium iodide staining and flow cytometry. Here, after an induction of 24 hr with 250 µM hydrogen peroxide, more apoptotic cells were detected in HEK APPsw as well as HEK APPwt cell populations compared to untransfected controls (Fig 3.40).

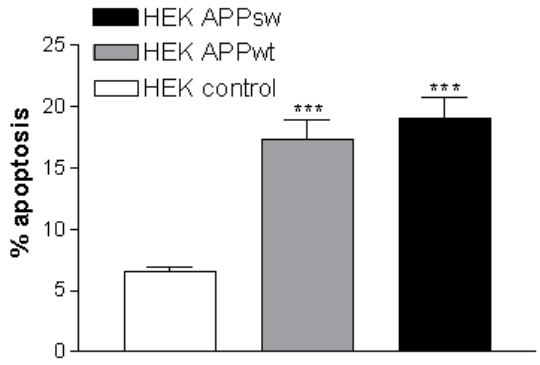

*Figure 3.40 Increase in apoptotic cells after treatment with H_2O_2 in HEK cells transfected with APPwt or APPsw compared to controls. Apoptotic cell death was assessed by propidium iodide staining and flow cytometry. (ANOVA ***p<0.001: ***p< 0.01 vs. control, posthoc Tukey's). Values are means ± SEM from 6-8 independent experiments.*

3.8.4 Effects of caspase inhibition in oxidative-stress induced cell death in HEK cells

Since HEK APPsw cells show increased apoptotic levels under normal culture contidions, caspase inhibitors were tested in their potential to inhibit the enhanced basal apoptosis of HEK APPsw cells, which produce high Aß levels. Therefore, cells were cultured for 24 hr in the presence of caspase inhibitors at a final concentration of 10 µM. As shown in Fig. 3.41 only the caspase 2 inhibitor was effective in reducing basal apoptotic levels of HEK APPsw cells. All other tested caspase inhibitors (caspase 3, caspase 8 and caspase 9) showed no protective effects. Thus, high Aß levels in HEK cells containing the Swedish APP mutation may lead to enhanced apoptosis through enhanced caspase 2 activation. In APPwt or untransfected HEK cells, no protective effect of caspase inhibitors could be detected.

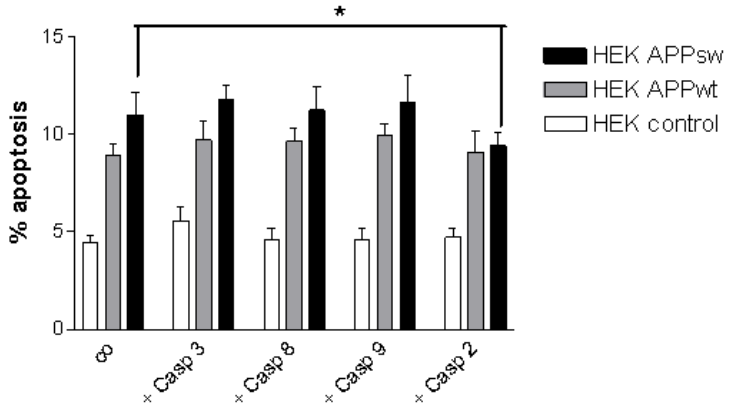

*Figure 3.41 Protective effects of caspase 2 inhibitor in basal apoptosis of APPsw transfected HEK cells (*p<0.05 vs. HEK APPsw co,student's t-test). No protection could be detected by caspase 3-, caspase 8- and caspase 9 inhibitor in basal apoptosis of HEK cells. Values are means ± SEM from 6-8 independent experiments.*

Caspase inhibitors were also tested in their potential to inhibit oxidative stress-induced apoptosis in HEK cells. As shown in Fig. 3.42 the pre-treatment of HEK cells with caspase 2 and caspase 3 inhibitors 3 hr prior the incubation with 250 µM hydrogen peroxide for 24 hr reduce significantly apoptotic levels in APPsw and APPwt cells, whereas no significant protection could be measured in untransfected controls.

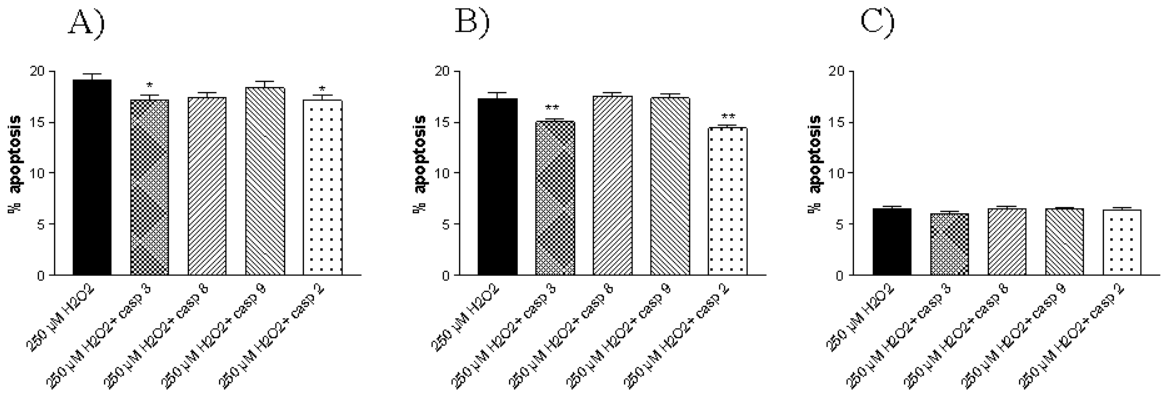

Figure 3.42 Caspase 2 and Caspase 3 inhibitor attenuates apoptosis significantly in APPSw transfected HEK cells (A) and in APPwt HEK cells (B). No protection could be detected by caspase 3-, caspase 8-, caspase 9 or caspase 2 inhibitor in oxidative stress induced apoptosis of untransfected HEK controls. Values are means ± SEM from 6-8 independent experiments.

3.8.5 JNK activation in HEK cells

Activation of JNK during oxidative stress induced cell death was also analyzed using an appropriate antibody recognizing only phoshorlyated JNK. As shown in Fig. 3.43, the incubation of HEK cells with hydrogen peroxide [250 µM] lead to a time dependent activation of JNK in the cytosolic fractions, whereas in both, APPwt and APPsw, JNK was found to be more activated compared to untransfected controls.

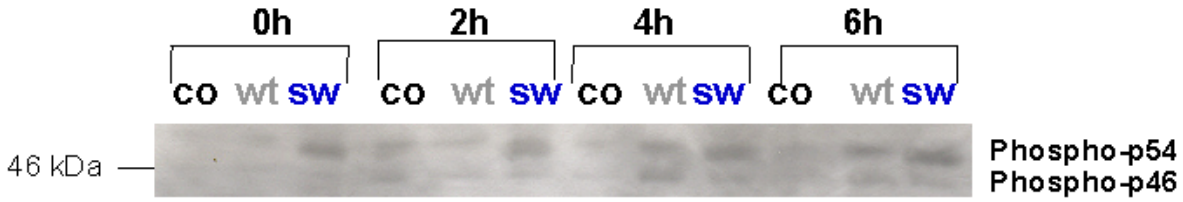

Figure 3.43 Activation of JNK during oxidative stress in cytosolic fraction of HEK cells Representative Western blotting is shown from three independent observations.

Analyzing the mitochondrial fractions, enhanced translocated phoshorylated JNK was found after 2 hr of oxidative stress induction, whereas both APP transfected HEK cell lines show more activated JNK in comparison to HEK controls. In particular, after 4 hr induction JNK activation is more pronounced in APPsw HEK.

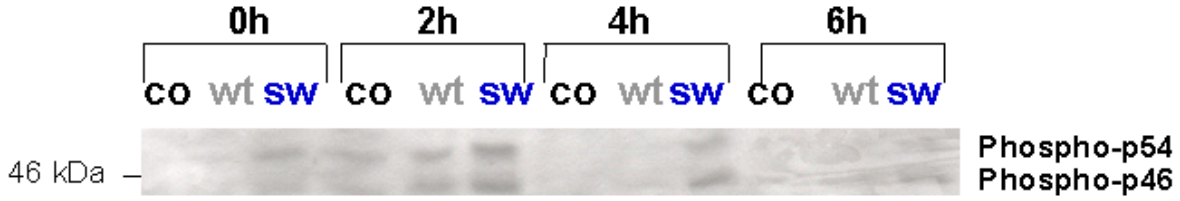

Figure 3.44 Translocation of JNK into mitochondrial fraction in HEK cells during oxidative stress. Representative Western blotting is shown from three independent observations.

3.8.6 Mitochondrial membrane potential

Since Mitochondrial membrane potential is a very important marker for the function of mitochondria and a decrease in mitochondrial membrane potential has been related to cell death in different cell types (Green 1998) the mitochondrial membrane potential of HEK was investigated. Interestingly, APPsw HEK cells showed a significantly decreased mitochondrial membrane potential in comparison to untransfected HEK cells and APPwt HEK cells (fig. 4xx). In comparison, APPsw bearing PC12 cells showed a slight hyperpolarised

mitochondrial membrane potential compared to vector cells and APPwt PC12 cells (Keil 2004). Treating PC12 cells with extracellular $A\beta_{1-42}$ results also in a significant depolarisation of mitochondrial membrane potential but to a lesser degree compared to APPsw HEK cells (Keil 2004). Thus, it is necessary to distinguish on the one hand between acute and chronic Aß effects and on the other hand between dose-dependent effects of Aß on mitochondrial function.

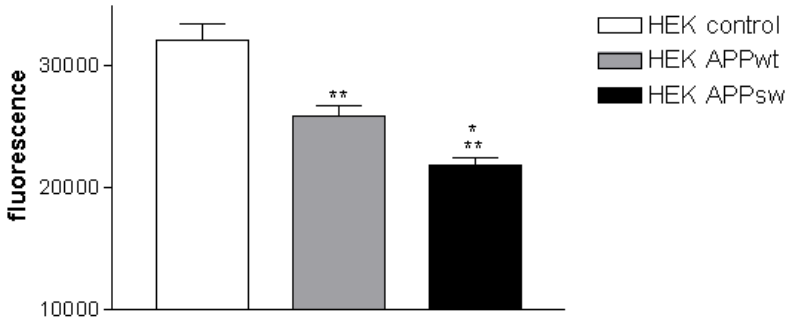

*Figure 3.45 Mitochondrial membrane potential is reduced in HEK cells containing the Swedish APP mutation compared to HEK APPwt or controls. (ANOVA ***p<0.001: ***p< 0.001 vs. control, *p < 0.05 vs. APPwt, posthoc Tukey's). Values are means ± SEM from 6 independent experiments.*

The effect of hydrogen peroxide on mitochondrial membrane potential of HEK cells was also investigated (Fig 3.46). A hydrogen peroxide incubation for 6 hr strongly reduces the mitochondrial membrane potential in a concentration dependent manner, showing that oxidative stress leads to mitochondrial damage in HEK cells.

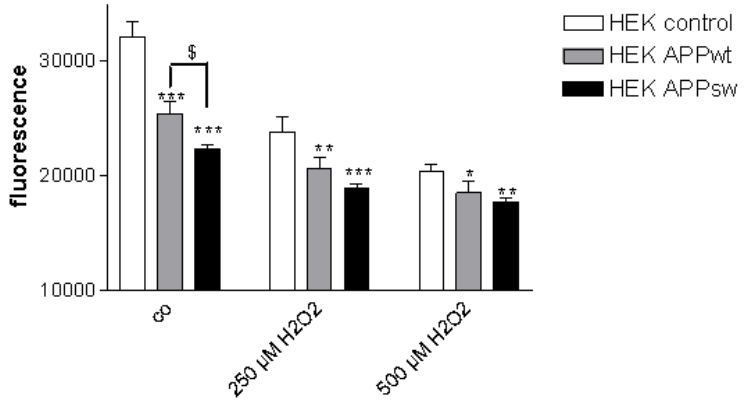

Figure 3.46 H_2O_2 reduce membrane potential in HEK cells in a concentration dependent manner. Values are means ± SEM from six independent experiments.

3.8.7 Effects of caspase 2 - and JNK inhibition on mitochondrial membrane potential in HEK cells

Similar as for PC12 cells, HEK cells were pre-treated with 200 nM JNK inhibitor SP600125 or 10 µM caspase 2 inhibitor before inducing oxidative stress. In contrast to PC12, neither JNK inhibitor nor caspase 2 inhibitor show protective effects in stabilizing mitochondrial membrane potential when oxidative stress is induced using 250 µM hydrogen peroxide. This difference to PC12 cells could be a result of the different cell types, leading to different mechanism in the mode of action of caspase 2. Unfortunately, due to a matter of time, the involvement of caspase 2 was not characterized more precisely in HEK cells.

Since JNK activation and translocation into mitochondria was observed in HEK cells (see 3.8.5), it would be assumable that JNK inhibitor may lead to protection of mitochondrial membrane potential, however, this could not be measured.

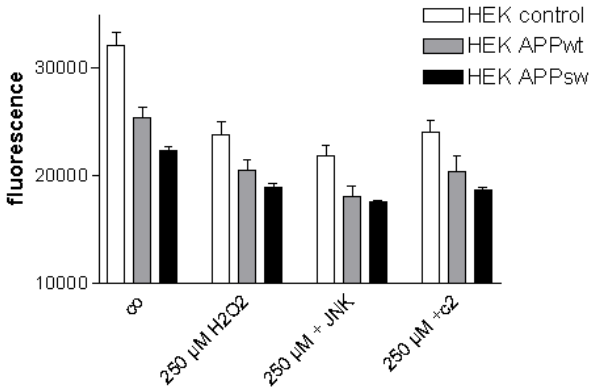

Figure 3.47 H_2O_2 reduced membrane potential in HEK cells. In contrast to PC12 cells, no protection of JNK- or caspase 2 inhibitor could be detected. Values are means ± SEM from 3-6 independent experiments.

3.8.8 ATP levels of APP transfected HEK cells

Since an intact membrane potential of mitochondria is needed to produce ATP in the mitochondrial respiratory chain, ATP levels were measured with an bioluminescence assay in addition to membrane potential. In accordance to the membrane potential, ATP levels were reduced in APPsw transfected HEK cells in comparison to APPwt and controls. Similar results have been obtained for APP transfected PC12 cells, but with lesser differences (Keil 2004), which leads the assumption that Aß has a dose-dependent effect on ATP levels. Extracellular Aß treated PC12 cells showed also reduced ATP levels (Keil 2004), but the ATP reduction was stronger under physiological conditions using APPsw PC12 cells compared to acute treatment with extracellular Aß (37% vs. 13%). Interestingly both, mitochondrial membrane potential and ATP levels in dissociated neurons of APP transgenic mice were also found to be reduced compared to littermate non-transgenic control mice (Keil 2004).

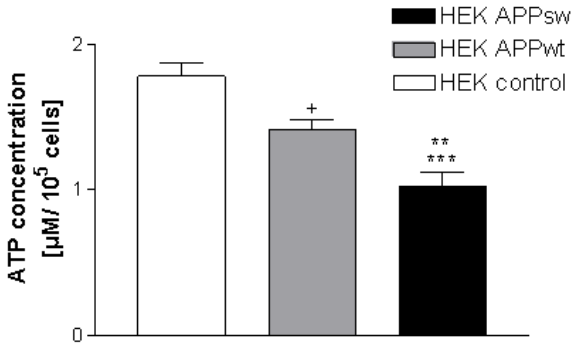

*Figure 3.48 ATP levels are reduced in HEK cells containing the Swedish APP mutation compared to HEK APPwt or controls, while (ANOVA ***p<0.001: **p< 0.01 vs. control, *p<0.001vs. APPwt, *p < 0.05 vs. control, posthoc Tukey's). Values are means ± SEM from 6 independent experiments.*

ATP levels of HEK cells were also measured after inducing oxidative stress. As shown in Fig. 3.49 ATP levels drop down already after a 30 min in a concentration dependent manner to hydrogen peroxide incubation.

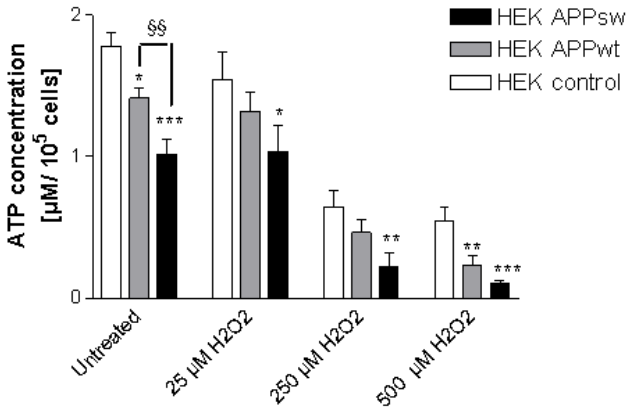

*Figure 3.49 After 30 min incubation with hydrogen peroxide ATP levels were strongly reduced in a concentration dependent manner. Two way ANOVA ***p <0,001 for H_2O_2-concentration dependent treatment and cell line,***p < 0,001,**p < 0,01 ,*p < 0,05 vs. control, $^{\$\$}p$ < 0,01 vs. APPwt, posthoc Tukey's. Values are means ± SEM from 3-6 independent experiments.*

3.8.9 Messenger-RNA expression of apoptotic factors

To further elucidate the involvement of other apoptotic factors in HEK cells, RT-PCR analysis was performed for AIF, GSK3ß and Presenilin 1. As shown in Fig. 3.50, and similar to PC12 cells, the induction of oxidative stress leads to an enhanced expression of mRNA of AIF, whereas in particular, HEK cells containing the Swedish APP mutation show a clearly upregulation of AIF mRNA expression. In addition, a clearly enhanced AIF protein expression was also found after 6 hr exposure to hydrogen peroxide in mitochondrial fractions of APPsw transfected HEK cells by Astrid Bonert from our group.

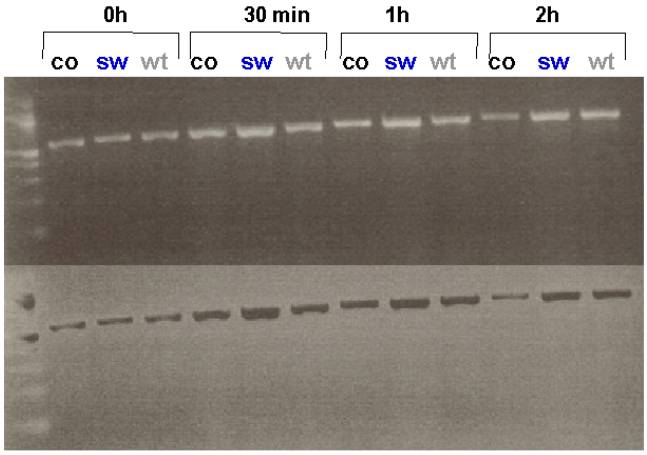

Fig. 3.50. Expression of AIF mRNA increases during oxidative stress in HEK cells, whereas cells containing the Swedish APP mutation show clearly enhanced mRNA expression in

comparison to APPwt or controls after 30 min H_2O_2 (250 µM) incubation. Data are from a representative experiment repeated at least three times with similar findings.

Interestingly, the release of AIF into the cytosol was only observed after an 24 hr hydrogen peroxide incubation, pointing for a role of AIF in a delayed apoptotic mechanism. As control, RT-PCR from the housekeeping gene ß-actin was performed from the same cDNA bank. Here, no alteration in the mRNA expression was detected.

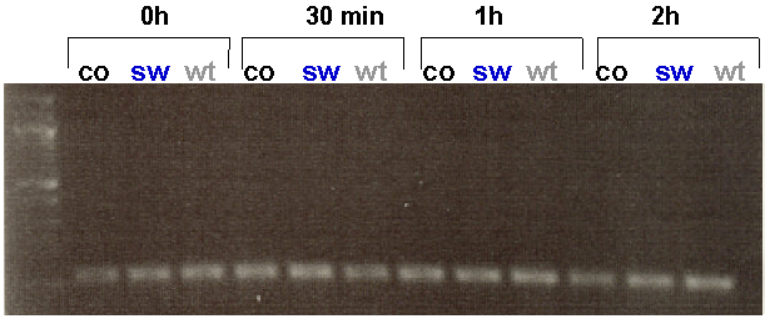

Fig. 3.51. ß-Actin mRNA expression in HEK cells. Data are from a representative experiment repeated at least three times with similar findings.

As already mentioned in 3.6.3 a very recent report by Ryder et al. (2004) hypothesized that Akt/GSK3ß mediated signalling may contribute to underlying AD pathogenesis induced by FAD mutations in APP and Presenilins. They found a down-regulated Akt/ PKB kinase activity and elevated endogenous GSK3ß kinase activity in HEK cells expressing the Swedish APP751. As shown in Fig. 3.52, the Swedish APP overexpression in HEK cells does not alter the mRNA expression of GSK3ß in HEK cells. Thus, rather postranslational modification or activation of GSK3ß may contribute to neurotoxicity than an upregulation in the mRNA levels of GSK3ß by the Swedish APP mutation. Nevertheless, enhanced mRNA expression of GSK3ß was found when oxidative stress was induced in HEK cells compared to untreated controls.

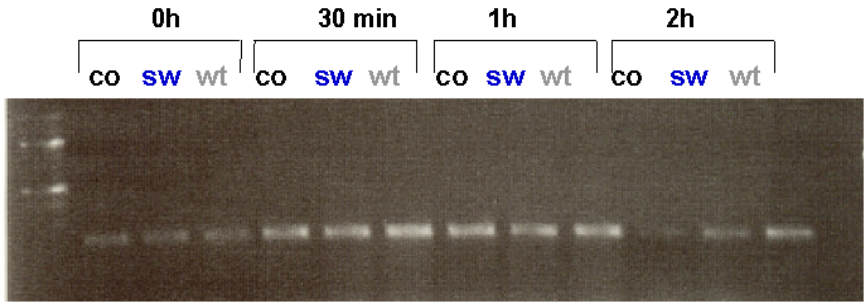

Fig. 3.52 Expression of GSK3ß mRNA in HEK cells increases during the induction of oxidative stress. Data are from a representative experiment repeated at least three times with similar findings.

Recently, Tamagno et al. (2002) showed that oxidative stress activates the expression and activity of BACE (see. 1.4.2), an aspartyl protease responsible for the beta-secretase cleavage of APP. It was also shown that APP containing the Swedish double mutation seems to be a significantly better substrate for β-secretase than wild-type APP (Citron 1992).

Presenilin 1 is an essential aspartly protease in the γ-secretase complex for the cleavage of APP (see. 1.4.3), and Aß production is enhanced by the Swedish mutation in HEK cells. Therefore, the mRNA expression of Presenilin1 in HEK cells was analysed, since primers for human Presenilin1 have been designed for the characterization of Presenilin transfected PC12 cells (see. 3.9.1) and were available in the lab.

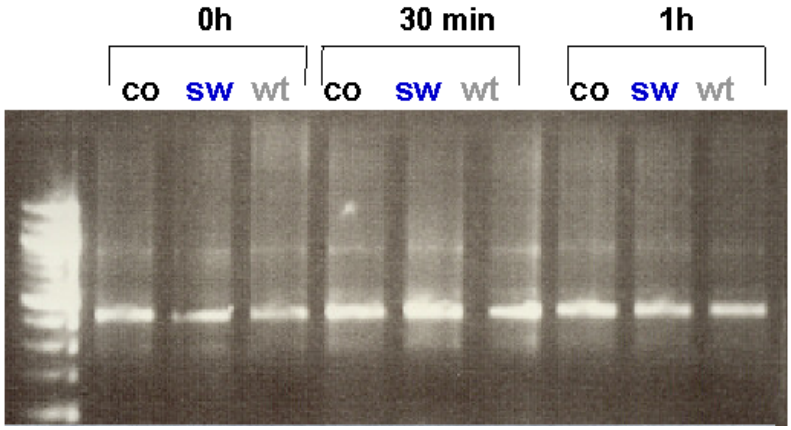

Fig. 3.52 The transfection of APP does not affect the Presenilin 1 mRNA expression in HEK cells. Data are from a representative experiment repeated at least three times with similar findings.

As shown in Fig. 3.52, the mRNA expression of Presenilin 1 in HEK cells is not altered by the APP overexpression, indicating that the enhanced Aß production in HEK APPsw cells may be rather through an enhanced activation of BACE (Kinoshota 2003) as a result of upregulated Presenilin 1. Nevertheless, the measurement of the γ-secretase activity or experiments using specific γ-secretase inhibitors, would further elucidate, whether altered γ-secretase contributes to the enhanced Aß production induced by the Swedish APP mutation.

3.9.1 APOPTOSIS IN CELL MODELLS OVEREXPRESSING PRESENILIN 1

Presenilin 1 (PS1) is an aspartyl protease, involved in the γ-secretase-mediated proteolysis of Aß. Recent studies have suggested an additional role of presenilin proteins in apoptotic cell death observed in AD. Since PS1 is proteolytically cleaved by caspase 3, it has been proposed that the resulting C-terminal fragment of PS1 (PSCas) could play a role in signal transduction during apoptosis. Moreover, it was shown that mutant presenilins causing early-onset of familial Alzheimer's disease (FAD) may render cells vulnerable to apoptosis. Recent experiments performed from Katharina Schindowski from our lab have revealed reduced apoptotic levels in $CD4^+$ T- lymphocytes of PS1wt transgenic mice compared to the littermate controls. In contrast, $CD4^+$ T- lymphocytes from mice bearing a FAD PS1 M146L mutation show increased vulnerability to cell death (Schindowski 2003).
The mechanism by which PS1 regulates apoptotic cell death is not yet understood. Therefore, one aim of this work was to clarify the involvement of PS1 in the proteolytic cascade of apoptosis and if the cleavage of PS1 by caspase 3 has a regulatory function.

3.9.1 Neuronal cell model

3.9.1.1 Characterization of PC12 cells expressing Presenilin

Starting from a pCDNA3 plasmid containing full length human PS1, two other plasmids, pCDNA3-PSCas and pCDNA3-PS1mut (D345A) were generated using appropriate oligonucleotides for PCR or mutagenesis (see.2.37). PC12 cells were transfected with respective pCDNA3-PS1, pCDNA3-PS1mut, pCDNA3-PSCas or empty pCDNA3 plasmids, and stably transfected cells were selected using G418 on the basis that pcDNA3-vector contains a neomycin- resistance cassette. The effective transfection of the different constructs were verified by RT-PCR (see. 2.3.6). One Primer pair was designed to amplify a 425 bp fragment of human PS1 from cDNA libraries of transfected PC12 cells, whereas the left primer starts at sequence size 956 while the complementary right primer recognizes PS1 starting at sequence size 1380. Since the constructed pcDNA3-PSCas contains only the C-terminal fragment of PS1 starting at the position 1048, another primer pair was designed to detect caspase 3 cleaved C-terminal fragment of PS1 from cDNA libraries of transfected PC12 cells. Here the left primer recognizes PS1 at sequence size 1180 while the complementary right primer starts at sequence size 1333, thus a 153 bp fragments is amplified

by this primer pair. As shown in Fig 3.53 an amplified fragment (sequence size 956 – 1380) was detected in Presenilin 1 (PS1) and mutated Presenilin (D345A, PSMut) transfected PC12 cells, while it was not detected in PC12 cells transfected with the caspase 3 cleaved C-terminal fragment of PS 1 (PSCas) or in empty vector transfected PC12 cells (control).

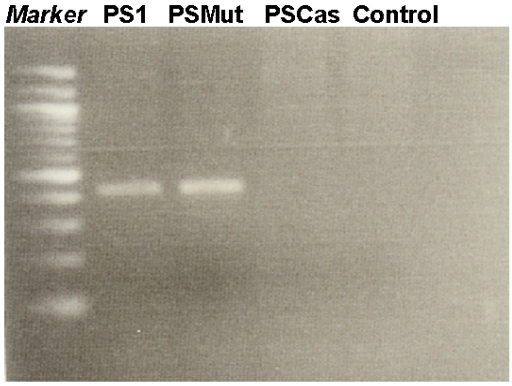

Fig: 3.53 RT-PCR analysis of human Presenilin 1 in transfected PC12 cells.

Using the second primer pair, which amplifies a fragment (sequence size 1180-1333) located near to the C-terminus of PS1, in all three human PS1 related cell lines (PS1, PSMut and PSCas) this 153 bp fragment was detected, showing that the respective transfected PC12 cells express human PS1 mRNA, while empty vector transfected cells show no mRNA expression of human PS1.

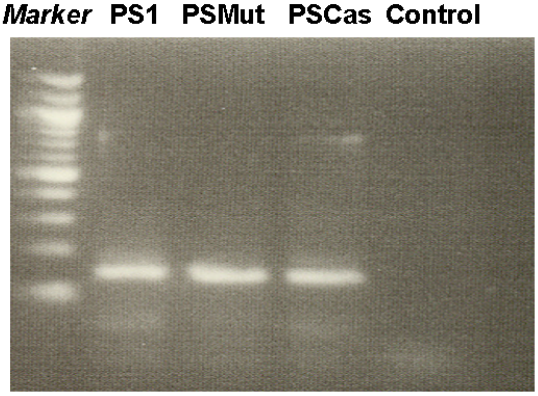

Fig: 3.53 RT-PCR analysis of human C-terminal Presenilin 1 in transfected PC12 cells.

3.9.1.2 Antiapoptotic effects of Presenilin during oxidative stress

Since PS1 is cleaved by caspase 3, generating a C-terminal fragment (PSCas), an involvement of PS1 in the regulation of apoptotic proteolytic cascade has been proposed (Kim 1997). The caspase-mediated cleavage of PS1 occurs between aspartate 345 and serine 346 (Grünberg et al, 1998).
Recent findings indicate that the expression of mutant PS1 (Hashimoto, 2002) or mutant APP (Eckert 2001) sensitises cultured cells to apoptosis. In addition to PS1, it has been shown that APP and PS2 are substrates of caspases *in vitro* and in cultured cells. (Gervais 1999, Kim 1997).
Despite these findings, it still remains unknown if PS1 has a direct regulatory function in apoptosis. Moreover, the function analyses of PS1 have been controversial. Due to pro-apoptotic reports of PS1, it has been hypothesized that caspase cleavage of presenilins and APP creates an apoptotic amplification cycle, accelerating cell death in the neurons of AD patients (Loetscher 1997; Kim 1997; Gervais 1999). In contrast, persuasive evidences describing an antiapoptotic action of PS1 have also been shown (Roperch 1998; Imafuku 1999; Amson 2000). PSCas and ALG-3, a truncated form of PS2, may inhibit Fas induced apoptosis (Vezina 1998; Vito 1997). PS1 assembles with the anti-apoptotic protein Bcl-2 into a macromolecular complex (Alberici 1999), and interacts with a novel presenilin-associated protein (PSAP) (XU 2002).

A negative regulation of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) signalling pathway by PS1 was proposed as the mechanism by which PS1 regulates apoptosis, since overexpressed PS1 suppressed the UV-stimulated SAPK/JNK activation in mouse embryonic fibroblast (MEF) from PS(+/+) mice.

To investigate the apoptotic effects of the overexpression of full length PS1, full lengths PS1 containing the mutated caspase 3 cleavage sequence site (PSmut, D345A) and the caspase 3 cleaved C-terminal fragment of Presenilin 1 (PSCas), transfected PC12 cells were incubated with 250 µM hydrogen peroxide for 24 hr, and apoptosis was measured by propidium iodide staining and flow cytometry. As shown in Fig. 3.54, the overexpression of full length PS1 as well as the caspase 3 cleaved C-terminal fragment of PS1 reduced apoptotic level when oxidative stress was induced in PC12 cell. However, the mutation at the caspase 3 recognition

site (D345A/ PSmut), which inhibits cleavage of PS1 by caspase 3, showed similar results as PS1 or PSCas towards oxidative stress stimuli in PC12 cells, showing also reduced apoptotic levels compared to empty vector transfected PC12 cells. This suggests that proteolysis of PS1 by caspase 3 may not be a determinant, but only a secondary effect during apoptosis.

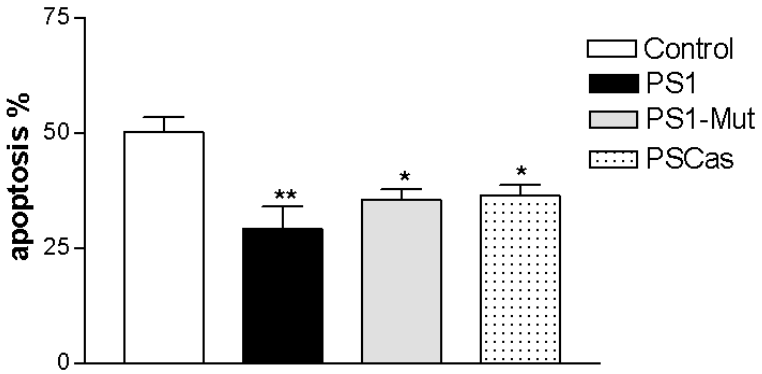

*Fig: 3.54 Oxidative-stress induced cell death in transfected PC12 cells. PS 1, PS1-Mut and PSCas transfected PC12 cells showed reduced sensitivity to oxidative stress-induced cell death compared to vector control cells (ANOVA, P < 0.01). Different from pCDNA3 vector control PC12 cells: posthoc Tukey's, **P<0.01 vs control, *P<0.05 vs control values are means ± SEM from 7 independent experiments.*

3.9.1.3 Presenilin and JNK activation

Since the overexpression of PS1, PSMut and PSCas reduce the sensitivity of PC12 cells to oxidative stress, it was investigated if the inhibition of the JNK pathway by PS1 might be a possible mechanism for its antiapoptotic action. A negative regulation of JNK signalling pathway has been observed recently by Kim et al. (2001). Exposure of HEK cells to UV light resulted in UV-stimulated SAPK/JNK activation, whereas this effect was reduced in transiently transfected HEK cells overexpressing PS1. In addition, mouse embryonic fibroblast (MEF) cells from PS1-null mice were more sensitive to hydrogen peroxide-induced apoptosis than in MEF cells from PS(+/+) mice (Kim 2001).

In order to investigate, whether the observed antiapoptotic effects of PS1, PSMut or PSCas in oxidative stress induced apoptosis in PC12 cells may be due to a negative regulation of JNK, western blotting of cell lysates from hydrogen peroxide treated PC12 cells was performed to detect activation of JNK. As shown in Fig. 3.55, the incubation of transfected PC12 cells

with hydrogen peroxide (6 hr, 250 µM) leads to activation of JNK, however, no reduction of the JNK activation could be observed, neither by the overexpression of PS1 and PSMut, nor by the overexpression of PSCas. Thus, in oxidative stress induced apoptosis in PC12 cells, the observed antiapoptotic mechanism may not be mediated through a negative regulation of JNK.

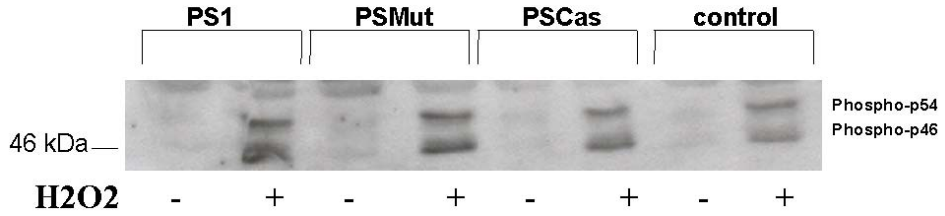

Fig: 3.55 Activation of JNK and c-Jun in oxidative stress-induced cell death in PS1, PSMut, PSCas and vector control transfected PC12 cells analyzed by Western blotting using anti-active p-JNK antibody. No reduction in the JNK activation was observed by the overexpression of PS1, PSMut or PSCas. Data are from a representative experiment repeated at least three times with similar findings.

3.9.1.4 Presenilin 1 overexpression does not effect mitochondrial membrane potential

As described in 1.5.3 apoptosis is inhibited by the antiapoptotic Bcl-2 involved in the regulation of apoptosis. Bcl-2 and its related proteins contribute to the formation of mitochondrial permeability transition pores. They regulate the release of calcium stores and control the release of mitochondrial factors from mitochondria to the cytosol. Using two-hybrid interaction system, and co-immunoprecipitation assays, Alberici et al. (1999) reported an interaction between PS1 and Bcl-2, and postulated that PS1 could have a role in apoptosis by providing a part of Bcl-2 protein in a macromolecular complex, and by liberating Bcl-2 from the complex in response to an apoptotic signal. It was hypothesized that PS1 may influence mitochondrial-dependent apoptotic activities, such as cytochrome *c* release and Bax-mediated apoptosis.

Before mitochondrial factors are released during apoptosis into the cytosol, a rapid reduction of the mitochondrial membrane occurs (Zamsami 1995). In the present study it was investigated whether the overexpression of PS1, PSMut or PSCas influences mitochondrial membrane potential. As shown in Fig. 3.56, no alteration in the basal mitochondrial

membrane potential in PS1, PSMut or PSCas transfected PC12 cells compared to vector transfected controls were observed. In addition, the incubation with hydrogen peroxide for 6 hr reduced membrane potential down to similar levels. Thus, no protection in stabilizing mitochodrial membrane potential by the overexpression of PS1, PSMut or PSCas could be detected.

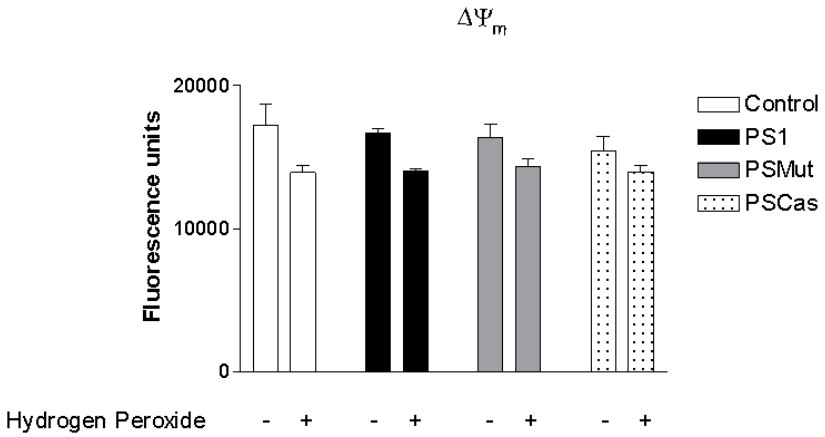

Fig: 3.56 mitochondrial membrane potential in PS1, PSMut, PS Cas and vector transfected PC12 cells. Values are means ± SEM from 6 independent experiments.

3.9.1.5 Presenilin and Bcl-xl expression

Bcl-xl is another anti-apoptotic member of the Bcl-2 family. Its expression is reduced in cytosolic fraction of APPwt and APPsw transfected PC12 cell (Keil 2004). Recently, it was shown by the yeast two-hybrid system that Bcl-xl interacts with the carboxyl-terminal fragments of PS1 and PS2 (Passer 1999). Interestingly, interaction analysis revealed that both PS2 and its naturally occurring carboxyl-terminal products, PS2short and PS2Cas, associated with Bcl-X_L, whereas the caspase-3-generated amino-terminal PS2NCas fragment did not. The authors proposed a role for Presenilins in modulating cell death by affecting Bcl-X_L activity.

Here, the bcl-xl expression was investigated, and as revealed by western blotting, no alteration in Bcl-xl expression was found by PS1 or PSCas in the cytosol of transfected PC12 cells compared to vector transfected controls. In addition, Bcl-xl expression was unchanged after incubation with peroxide incubation (250 µM, 24 hr), confirming results of Astrid Bonert from our group.

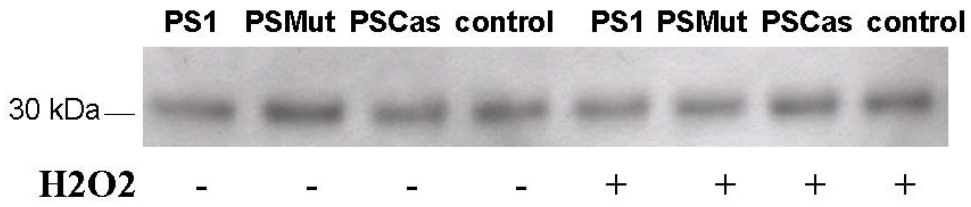

Fig: 3.57 Bcl-xl expression is unaltered by Presenilin 1 overexpression in PC12 cells

Since none of the examined apoptotic factors (JNK, mitochondrial membrane potential and Bcl xl expression) seem to be involved in the antiapoptotic action of PS1, other factors need to be considered.

Recent work indicates that PS1 interacts with glycogen synthase kinase 3beta (GSK3beta). *In vivo*, GSK3beta phosphorylates kinesin light chains (KLC) and causes the release of kinesin-I from membrane-bound organelles (MBOs), leading to a reduction in kinesin-I driven motility (Morfini 2002b). These findings suggest that mutations in PS1 may compromise neuronal function by affecting GSK-3 activity and kinesin-I-based motility.

Thus, it is possible that in PC12 cells transfected with human PS1, the interaction of overexpressed PS1 with GSK3ß could lead to reduced GSK3beta activation, since it has been shown that GSK3beta activity were increased in fibroblast cultures cells of embyronic knockout PS1 (PS1-/-) mice (Pigino 2003). Therefore, analysis of the GSK3ß activation are currently under investigation.

3.9.1.6 Vulnerability against Staurosporine-induced apoptotis

In addition to hydrogen peroxide, a second apoptotic stimulation was used to analyse the role of PS1 in apoptosis. Staurosporine is an alkaloid produced by *Streptomyces* bacteria. It is known to be a broad-spectrum kinase inhibitor with greate potency and is described as a Protein Kinase C (PKC) inhibitor (Tamaoki 1986). The exact mechanism of its action is still unclear, an activation of several caspases has been observed (Massieu 2004). Nevertheless, staurosporine may also induce apoptosis in a caspase-independent pathway (Zhang 2004) Additionally, staurosporine-induced neuronal death is associated with increased production of reactive oxygen species (Krohn 1998). Recently, Steffen Leutz from our lab ascertained that a

reduction of trophic support enhance apoptosis in APPsw transfected PC12 cells (Leutz 2002). Here it was investigated, whether the overexpression of PS1, PSMut or PSCas also leads to reduced apoptotic levels in staurosporine-induced cell death compared to vector transfected controls. Therefore, PC12 cells were incubated with 500 nM staurosporine for 24 hr, and apoptosis measured by propidium iodide staining and flow cytometry.

In contrast to oxidative stress-induced apoptosis, PC12 cells containing human PS1, PS1Mut or PSCas show no significant reduction in apoptotic levels compared to empty vector transfected controls. Only a tendency in reducing apoptosis could be detected. This difference to oxidative stress induced cell death could be a result of the different apoptotic mechanism of oxidative stress and staurosporine. Since Zhang et al. (2004) observed a caspase independent apoptosis and late apoptotic execution of apoptosis induced by staurosporine, other apoptotic factors may rather contribute to apoptosis, that could not be affected by Presenilins.

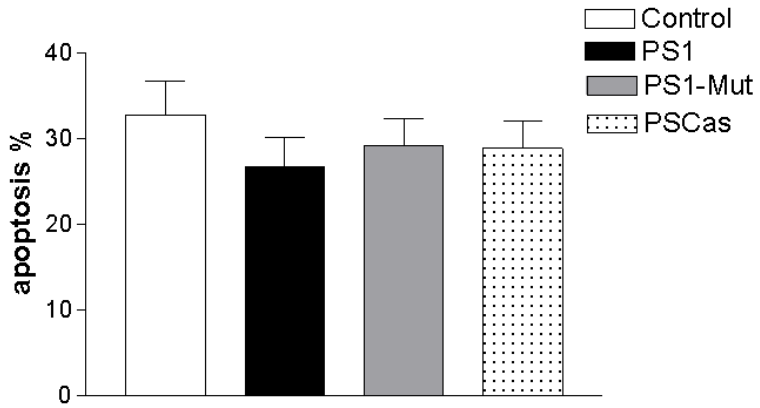

Fig: 3.58 Apoptotic cell death in PC12 cells induced by 500 nM staurosporine. Values are means ± SEM from 5 independent experiments.

Interestingly in this context is that the Swedish APP mutation sensitises PC12 cells to staurosporine-induced cell death only when at the same time the trophic support was reduced. If PS1 overexpression has antiapoptotic effects in PC12 cells under these conditions still needs to be verified.

3.9.2 Peripheral cell model

The use of peripheral Alzheimer cells is a common application to study mechanisms involved in the disease. Lymphocyte cell lines, transfected with mutant PS1 (Wolozin 1998), and

lymphocytes from FAD patients bearing PS1 mutations (Parshad 1996) show similar results to primary neurons from FAD mutant mice (Guo 1999) with regard to an increased vulnerability to apoptosis. Recently, it was shown that CD4+ T-lymphocytes affected by both, sporadic or genetic APP and PS1 AD risk factors share an increased vulnerability to cell death (Schindowski 2003). In lymphocytes from PS1wt-transgenic mice, it was observed that spontaneous apoptosis is significantly reduced in $CD4^+$ T-cells from PS1wt-transgenic mice compared to non-transgenic littermate controls. Thus, lymphocytes may serve as a cellular system in which to study risk factors of sporadic, as well as genetic AD *in vivo*.

3.9.2.1 Fas induced apoptosis in Jurkat cells

In previous experiments made by in our lab several constructs encoding full-length PS2, ALG-3 (a truncated C-terminal fragment of PS2) were made and transfected into Jurkat T-cell lymphoma cells. The results indicate a clearly reduced Fas-ligand-induced apoptosis by the C-terminal fragment of PS2.

In order to investigate a possible functional role of PS1 in the proteolytic cascade of apoptosis, similar experiments were performed transfecting Jurkat cells transiently with PS1, PSMut and PSCas and inducing apoptosis with 50 ng/ml Fas-ligand for 24 hr. Here, Fas-ligand induced apoptosis was reduced by PS1, PSMut or PSCas in transiently transfected Jurkat cells.

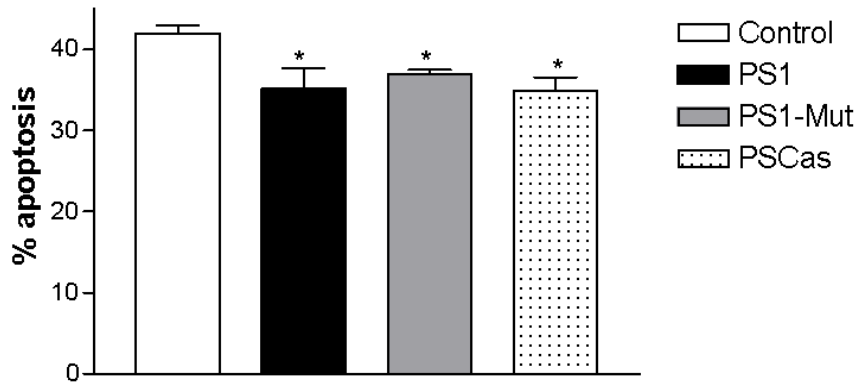

*Fig: 3.59 Apoptotic cell death induced by 50 ng/ml Fas ligand in transiently transfected Jurkat cells. Apoptosis was measured after 24 hr by PI-staining and FACS anaylsis. (ANOVA, $P < 0.05$). Different from pCDNA3 vector control transfected Jurkat cells: posthoc Tukey's, *$p<0.05$ vs control. Values are means ± SEM from 5 independent experiments.*

3.9.2.2 Staurosporine induced apoptosis in Jurkat cells

In addition to the induction of apoptosis by Fas ligand, apoptotic levels of transiently transfected Jurkat cells were also measured in staurosporine-induced cell death. Here no antiapoptotic effect could be ascertained as a result of the overexpression of PS1, PSMut or PSCas. Thus, the antiapoptotic effect may be dependent on the apoptotic stimulus.

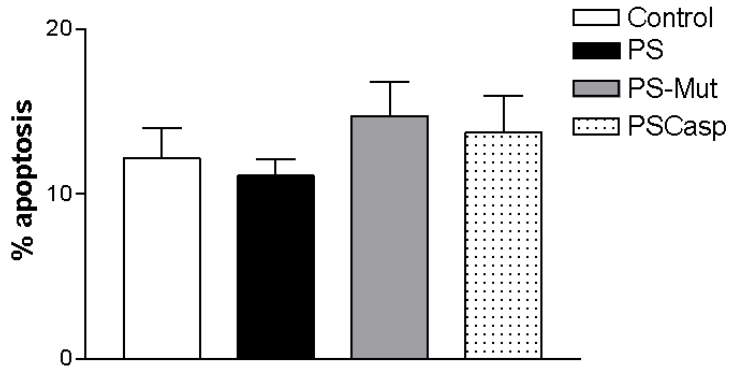

Fig: 3.60 Apoptotic cell death in PC12 cells induced by 500 nM Staurosporine in transiently transfected Jurkat cells. Values are means ± SEM from four to six independent experiments

4. Discussion

4.1 APPSW MUTATION LEADS TO AN ENHANCED VULNERABILITY TO OXIDATIVE STRESS-INDUCED APOPTOSIS IN PC12 CELLS

The present thesis demonstrates that the overexpression of the familial Alzheimer's disease-related Swedish APP mutation, which is located near the N-terminus of the Aß region, can increase the vulnerability to oxidative stress induced cell death in PC12 cells. This death mechanism is probably initiated by intracellulary produced Aß.

Though Aß-induced cell death has been a topic of research in many studies, the relevance of these *in vitro* findings with regard to *in vivo* processes is not clear. In most studies, synthetic Aß peptides have been applied at micromolar levels that are in contrast to the low nanomolar levels of natural Aß found in the brain and cerebrospinal fluid of AD patients. Therefore, studies of Aß-induced neurotoxic effects under more likely physiological conditions mimicking the situation in AD brains are needed (Selkoe 2002). Moreover, Aß is produced intracellulary and can accumulate within cells (Wirths 2001; Hartmann 1997; Hartmann 1999) before its deposition in senile plaques. Intracellular accumulation of Aß might impair cellular functions and may probably represent the primary event within the neurotoxic Aß cascade.

In order to mimic the situation *in vivo*, PC12 cells were transfected with human APP and the FAD-related Swedish APP-Mutation. APPsw and APPwt PC12 cells showed a moderate expression of human APP leading to low levels of Aß(1-40) production (APPsw: 90 pg/ml; APPwt 20 pg/ml) similar to the situation *in vivo*. These two cell lines with different production levels of Aß may additionally allow to study dose-dependent effects of Aß.

Interestingly, in the absence of additional apoptosis-inducing treatments, no evidence has been found for increased apoptosis in APPsw transfected PC12 cells compared to APPwt cells or control cells (Eckert 2001). This is consistent with findings in differentiated neuroblastoma B103 cells bearing mutant APP V717I (Xu 1999) or in other cell lines, e.g., PC12 cells (Guo 1997; Keller 1998) or neuroblastoma cells (Tanii 2000), both with PS-1 mutations, indicating that the expression of moderate levels of the mutant variant does not trigger enhanced apoptosis per se.

Therefore, it seems rather likely that increased production of Aß at physiological levels primes APPsw cells to undergo death, leading to increased cell death only after a secondary insult, e.g., oxidative stress. This scenario which has been suggested to occur in AD brain might explain the early onset form of Alzheimer's disease in the rare familial cases, in which mutations in the APP gene lead to an enhanced Aß production and a 100 % penetrance of the disease. This hypothesis is supported by the fact that the brain has a high metabolic rate and is exposed to gradually rising levels of oxidative stress during life and that in Swedish FAD patients the levels of oxidative stress are increased in the temporal inferior cortex (Bogdanovic 2001).

This study using a cell model mimicking the *in vivo* situation in AD brains indicates that probably both, increased Aß production and the gradual rise of oxidative stress throughout life, lead to an increased vulnerability of neurons to apoptotic cell death in FAD patients.

4.2 CASPASE ACTIVATION IN RESPONSE TO OXIDATIVE STRESS-INDUCED CELL DEATH

The caspase family of enzymes is a large group of proteases, whose members have defined roles in apoptotic cell death (Thornberry 1998). These enzymes can be subdivided according to the feature of their prodomains into two groups: initiator caspases, which are usually the first to be activated in the proteolytic cascade of apoptosis and effector caspases, which are activated by initiator caspases, and in turn execute apoptotic cell death by cleaving crucial cellular substrates (see. 1.5.3).
Since caspases play a central role in the regulation of apoptotic signalling, the involvement of several caspases in oxidative stress-induced apoptosis was investigated to elucidate the mechanism leading to enhanced vulnerability of PC12 cells containing the Swedish APP mutation.

4.2.1 The Swedish APP mutation induces activation of caspase 2 in PC12 cells

Caspase 2 was initially described as a neuronally expressed caspase that was down regulated during the course of brain development (Kumar 1994) and although caspase 2 was the second mammalian caspase described (Kumar 1994), it has not generated the sustained interest that

has been focused on other members of the caspase family such as caspase 3, -8 and -9. Only in the last years this changed because of studies that have shown a role of caspase 2 in permeabilizing mitochondria (Lassus 2002; Paroni 2002; Robertsen 2002).

Using western blot analysis and a kinetic colorimetric assay measuring the enzyme activity, it was observed that in PC12 cells an activation of caspase 2 in oxidative stress signalling occured. In APPsw cells, the maximal caspase 2 activity was fourfold higher compared to APPwt and vector PC12 cells, respectively.

How does caspase 2 activation occur in response to oxidative stress in PC12 cells?

Today it is still not clear which mechanism leads to activation of caspase 2. A study reporting the identification of an adaptor protein for caspase 2, RAIDD, suggested that caspase 2 can be activated in TNFR-mediated cell death (Duan 1997). These report showed that RAIDD interacts with RIP, then with TNFR1 via TRADD, and presumably recruits caspase 2 to this complex to carry out TNFR1-mediated cell death. However, it is not clear that RAIDD is always required for the activation of caspase 2. While the overexpression of RAIDD can lead to cell death, blocking RAIDD or caspase 2 with dominant-negative forms did not abrogate TNF-mediated cell death, thus the relevance of RAIDD and/or caspase 2 in receptor-induced death is unknown.

Interestingly, the analysis of the primary sequence of caspase 2 revealed that the prodomain of caspase 2 is more closely related in structure to that of caspase 9 than of caspase 8, which is activated through interaction of its death effector domain with FADD in extrinsic apoptotic pathway (see.1.5.3.1). Both, caspase 9 and caspase 2 contain CARD (caspase recruitment domain) domains (Lamkafi 2002). Caspase 9 it is known to be activated by apoptosome formation. Thus, it was proposed that caspase 2 may also be activated in an apoptosome. A novel reported PACAP protein (proapoptotic caspase adaptor protein) that interacts with caspase 2 and 9, might be responsible for the formation of a caspase 2 apoptosome (Bonfoco 2001). More recently, it was reported that caspase 2 is recruited to a large protein complex of over 670 kDa independent of cytochrome c and Apaf-1 and that this recruitment is sufficient for caspase 2 activation (Read 2002), whereas this activation occurs before cytochrome c is release into the cytosol.

Since only little it is known about the mode of caspase 2 activation, it is also difficult to address, if the caspase 2 activation in oxidative stress-induced apoptosis in PC12 cells may be due to an large protein complex formation and/or through receptor induced death and RAIDD interaction. It is also possible that RAIDD could be a component of this large protein complex which activates caspase 2. The elucidation of this large protein complex structure and the identification of its components will help to delineate the molecular mechanism of caspase 2 activation.

More evidence for caspase 2 activation by RAIDD interaction comes from a very recent report (Jabado 2004) showing that rat RAIDD, cloned from PC12 cells, interacts with caspase 2 CARD, whereas overexpression of RAIDD induced caspase 2- and caspase 9-dependent apoptosis of PC12 cells. In experiments using trophic deprivation, RAIDD overexpression increased cell death and formation of RAIDD aggregates. Thus in PC12 cells, an enhanced formation of RAIDD aggregates in oxidative stress, similar to trophic factor withdrawal-induced apoptosis, is possible.

Enhanced caspase 2 activation in APPsw transfected PC12 cells

In addition to the mode of activation, only little is known about the function of caspase 2 in death pathways. Caspase 2 has been found to mediate trophic factor deprivation death in several different cell types, including PC12 cells (Troy 1997) and primary cultures of sympathetic neurons (Troy 2002), whereas caspase 2 seems to act independent of caspase 3 activation.

Recent reports indicate procaspase 2 localized in mitochondria (Susin 1999) and that mitochondria contain oxidative stress induced caspase 2 activity (Takahashi 2004). Depending on experimental conditions, in our study a caspase 2 inhibitor was able to protect PC12 cells from the collapse of the mitochondrial membrane potential (see.3.25). The relationship between caspase 2 activation and oxidative stress-induced alterations in mitochondria is not clear at present. A connection could exist in alteration of the pH gradient in mitochondria. According to Mitchel (1996), during mitochondrial respiration, protons are pumped outwards across the innermitochondrial membrane, generating a proton-motive force (Δp), across this membrane. This electrochemical gradient consists of the inner mitochondrial

membrane potential (Δψm, negative inside) and a pH gradient (Δ *pH*m, alkaline inside). At physiological temperature the following equation is asserted:

$$\Delta p = \Delta\psi m - 60\Delta pHm.$$

The regulation of Δψm and Δ*pH*m is tightly coupled and the efficiency of ATP synthesis and other mitochondrial physiology (e.g. Na+/Ca2+ exchange, K+/H+ antiport) is very sensitive to the mitochondrial pH gradient.

New findings of Takahashi et al (2004) show that oxidative stress induces a decrease of the alkaline mitochondrial pH, whereas the antiapoptotic Bcl-2 may exert its protective activity via modulation of mitochondrial pH. In addition, Guo et al. (2002) observed that Bcl-2 and Bcl-xL can block caspase 2-induced cell death. Further experiments analysing the effects of caspase 2 inhibitor on mitochondrial pH gradient will elucidate, whether caspase 2 inhibitors exert their protective effect on mitochondrial membrane potential through stabilization of the mitochondrial pH gradient. Also it will be helpful to analyse, whether an increased Bcl-2 or Bcl-xl expression has similar effects. In this context it is interestingly that APPsw transfected PC12 cells have reduced expression of Bcl-xl under basal conditions and after exposure to hydrogen peroxide (Bonert 2004).

In addition to mitochondria, caspase 2 was found to be localized in several other intracellular compartments, including Golgi, cytosol, and nucleus (Mancini 2001; Paroni 2002; Zhivotovsky 1999). Cytotoxic stress causes activation of caspase 2, which is required for the permeabilization of mitochondria (Lassus 2002), and induces the release of cytochrome c (Paroni 2002, Robertson 2002, Guo 2002).

It is assumable that the enhanced Aß production in the APPsw transfected cells occurs through an increased expression and activity of BACE (Tamagno 2002). The elevated intracellular levels of Aß in the mutant cells may therefore lead to an enhanced activation of caspase 2, which in turn implements the activation of the intrinsic apoptotic pathway by permeabilizing mitochondria and reducing mitochondrial pH. Since caspase 2 activation occurs early in our cell model and neurons from caspase 2 null mice are resistant to ß-amyloid-induced death (Troy 2000) this caspase seems to play a critical role in ß-amyloid toxicity.

This assumption is supported by experiments measuring the membrane potential of PC12 cells pre-treated with caspase 2 inhibitor VDVAD-FMK. Under certain experimental conditions a protective effect of caspase 2 inhibitor on mitochondrial membrane potential was observed in oxidative stress induced apoptosis. This effect was specifically enhanced in PC12

cells transfected with the Swedish APP mutation (see. 3.3.4). Moreover, in APPsw transfected HEK cells, which exhibit a 30-fold increased Aß production compared to APPsw bearing PC12 cells, the enhanced basal apoptotic levels were reduced by the caspase 2 inhibitor. Since it has also been reported that caspase 2 induces a caspase 3 independent apoptotic pathway (Troy 2000), other effects of caspase 2 need to be considered. It is still unknown, whether caspase 2 also induces the release of AIF to cytosol, or if a nonmitochondrial apoptotic pathway is induced by ß-amyloid through enhanced caspase 2 activation.

4.2.2 Enhanced caspase 8 activation by the Swedish APP mutation during oxidative stress

As described in 1.5.3.1 apoptosis can be triggered by different apoptotic stimuli, and two different apoptotic pathways have been described. During the intrinsic apoptotic pathway diverse apoptotic signals converge at mitochondrial level, pointing a pivotal role of mitochondria in regulating this apoptotic pathway. The other well described pathway is termed the extrinsic apoptotic pathway. Here, various death receptors have been described, which belong to the TNF superfamily of receptors and trigger external signals into the cell by binding specific ligands. These death receptors, including CD95/Fas, DR4 and DR5 (death receptors 4 and 5; also called TRAIL-R1 and TRAIL-R2), as well as TNFR1 and are activated by their cognate ligands, CD95 ligand, TRAIL (TNF-related apoptosis-inducing ligand) and TNF, respectively.
Each of these receptors have in common that they activate caspase 8 by first recruiting the adapter proteins, FADD (Fas-associated death domain) and/or TRADD (TNF receptor-associated death domain). In particular, the complex of CD95 with FADD and caspase 8 is referred as the DISC or death-inducing signalling complex. Following its activation within the complex, processed caspase 8 can in turn activate the effector caspase 3, which cleaves critical structural and regulatory proteins, resulting in apoptosis.

Since PC12 cells transfected with the Swedish APP mutation showed enhanced vulnerability in response to oxidative stress induced apoptosis, it was examined whether the initiator caspase 8 is involved. Therefore, the expression pf activated caspase 8 and caspase 8 activity were measured The results show clearly that after oxidative stress the caspase 8 activity increased in PC12 cells with the highest activities measured for APPsw transfected cells after 2 hr hydrogen peroxide incubation. This was not implicitly expected, since it has been

described that oxidative stress induces intrinsic the apoptotic pathway (Halliwell 1994; Lotem 1996; Pias 2002). In vector transfected control cells, in fact the caspase 8 activity increased only slightly. Compared to the basal activity, the maximal caspase 8 activity in vector transfected PC12 cells did not even duplicated, while the maximal caspase 8 activity in APPsw transfected PC12 cells was about fourfold higher as the basal activity. In addition, the caspase 8 activity was higher in APPsw cells compared to APPwt cells.

How is caspase 8 involved in oxidative stress induced apoptosis in APP harbouring PC12 cells?

The obtained results show that the additional caspase 8 activation could specifically contribute to the enhanced vulnerability in APPsw transfected PC12 cells, as measured by propidium iodide staining and FACS analysis. However, an important question that needs to be addressed, is how the Swedish APP mutation leads to the enhanced caspase 8 activation? Since PC12 cells harbouring the Swedish APP mutation produce more Aß, it is attempting to assume that the enhanced Aß levels might be responsible for this caspase 8 activation. In addition to the data obtained in this thesis, there is further evidence pointing caspase 8 to be involved in AD pathology. A very interesting recent report shows that soluble Aß might interact with APP leading to an APP-dimer complex formation (Lu 2003). This generated APP complex may induce toxicity similar to other known physiological pathways, such as Fas ligand induced cell death. Similar to TNF receptor, the multimerization of APP by Aß recruits caspase 8 to the APP complex, leading to activation of caspase 8. Thus, it is possible that enhanced Aß production in PC12 cells could lead to enhanced caspase 8 activation by this mechanism. This effect might be enhanced by an additional oxidative stress insult.
That APP itself could act as death receptor is also suggested by experiments adding an antibody to the extracellular region of APP which results in apoptosis. This effect was possibly mediated by cross-linking APP, because the effect was seen with bivalent but not monovalent antibody (Rohn 2000; Sudo 2000). Interestingly, in a other recent report the dimerizated APP induces apoptosis via its cytoplasmic domain by activating JNK (Hashimoto 2003), an effect, that was also observed by the Swedish APP mutation.

Furthermore, it has also been shown that caspase 8 is not only activated by Aß, but is also implicated in an alternative processing of APP during apoptosis (Pelligrini 1999). Since APP contains a caspase cleavage site with the essential aspartate at position 664, a resulting C31 APP fragment was generated by *in vitro* cleavage assays from APP, while caspase 8 inhibitor and mutagenesis of APP aborted the formation of C31. In addition to caspase 8, it was also proposed that APP is cleaved by caspase 6 (Gervais 1999), whereas the Swedish mutated APP may present a better caspase 6 cleavage sequence (VNLD, with the aspartate at position 672) as wildtyp APP (VKMD). However, experiments measuring caspase 6 activity in PC12 cells revealed no induction of caspase 6 by the Swedish APP mutation (Marques 2001, diploma thesis).
It was also reported that the caspase 8 cleaved APP fragment C31 may have toxic properties (Lu 2000; Nishimura 2002) contributing to neuronal cell death. The recent findings that in addition to APP-dimer complex formation, Aß also induces the C31 generation by caspase 8 cleavage of APP in the C-terminus (Lu 2003b) suggest that Aß-mediated toxicity initiates a cascade that includes caspase activation and APP cleavage.

Taken together, our findings and the results obtained by other groups, it can be hypothesized that the overproduction of Aß in APPsw PC12 cells leads to APP cleavage and APP-dimer complex formation. The enhanced vulnerability of APPsw transfected PC12 cells could be a result of the recruitment of caspase 8 by the APP complex and an additional toxic effect of C31. Nevertheless, this hypothesis needs to be confirmed by experiments, for example using a C-terminal antibody against APP that is also able to recognize C31 in PC12 cells undergoing apoptosis, or trying to co-immunoprecipitate caspase 8 with APP. Since it has also been shown that oxidative stress induce the increase of intracellular Aß (Misonou 2000), the additional hydrogen peroxide incubation could lead to increased Aß production, emphazing the caspase 8 activation. Other death receptors recruit adapter proteins like FADD to bind the death effector domain of caspase 8. Therefore, it should also be examined, whether the APP complex recruit FADD or other adapter proteins, or if the caspase 8 is direct associated to APP.

Another possibility has also to be considered to explain the enhanced caspase 8 activity in APPsw PC12 cells. The involvement of other death receptors in Aß toxicity needs to be assumed as proposed recently by Cantarella et al. (2003), describing the implication of TNF-related apoptosis-inducing ligand (TRAIL) in Aß-mediated neurotoxicity in human SH-SY5Y

neuronal cells. The mRNA and protein expression of TRAIL was found to be increased when cells were treated with Aß. TRAIL was released into the culture medium and found to have neurotoxic properties by inducing caspase 8 activation, whereas the overexpression dominant negative FADD and caspase 8 inhibitor rescued cells.
Very recently, a neuronal response of Aß occuring through a TNF receptor signalling cascade and a caspase-dependent death pathway has been proposed (Li 2004). Neurons in both AD brain and Aß-treated cultures exhibited Fas-ligand upregulation and changes in immunoreactivity for Fas receptor (Su 2003). In this context it is interesting that hydrogen peroxide upregulates Fas and Fas-ligand expression in PC12 cells (Facchinetti 2002). Using concentrations of 250 µM and 400 µM hydrogen peroxide, Fas and FasL mRNA levels increased rapidly. Hence it will be interesting to analyse whether the enhanced caspase 8 activity in APPsw cells could be a result of upregulated Fas, TNF or Trail expression.

4.2.3 Oxidative stress induced mitochondrial dysfunction leading to caspase 9 activation

The investigation of the caspase 9 involvement revealed that the induction of oxidative stress led to an increased caspase 9 activity in APPwt PC12 cells as well as in APPsw PC12 cells compared to vector transfected control cells. At the same time an enhanced cytochrome c release has been detected in both, APPsw and APPwt compared to vector control (Marques 2003). For the interpretation of these results, the understanding of the pivotal role of mitochondria in regulating cell death and energy metabolism is very important, since it is not known, how AD-related genetic factors are linked to oxidative stress.

Mitochondrial damage induced by overexpression of APP

The respiratory chain reaction in mitochondria inevitably generates reactive oxygen species (ROS) including oxygen radicals and hydrogen peroxide. When the ROS production dominates cellular ROS-reducing functions, oxidative stress develops (see 1.6.1), ultimately leading to apoptosis. At the same time, mitochondria serve as the reservoir of death signals originating from physiological stresses, controlling the release of several apoptotic factors like cytochrome c (see 1.5.3.2). Before cytochrome c is released from the intermembrane space into the cytosol, mitochondria need to disrupt the inner membrane potential (Green 1998). The adenine nucleotide translocator (Vieira 2000; Belzacq 2001) and the voltage-dependent

anion channel (VDAC) (Shimizu 1999; Madesh 2001) are thought to accomplish the cytochrome *c* translocation under the control of Bcl-2 family proteins (Belzacq 2003). However, it has also been reported that pro-apoptotic 'BH3-only' molecules, tBID, and Bik, can release cytochrome c by outer membrane pore formation in cooperation with the 'BH1-3' members represented by Bax and Bak (Wei 2001).

Thus, analyzing the pivotal role of mitochondria in oxidative stress induced apoptosis has been one main topic of research in our lab. The obtained results show that the expression of APPsw leads to a reduction of the cytochrome c oxidase activity (complex IV of the mitochondrial respiratory chain) as well as an impairment of complex II, III and V (Keil 2004). In this connection it has been observed that basal ATP levels of APPsw PC12 cells were significantly reduced compared to vector transfected control cells. APPwt PC12 cells show also reduced ATP levels in comparison to vector trasnfected PC12 cells but to a lesser extent than APPsw.

How to explain the effects of the Swedish APP mutation on mitochondria in PC12 cells?

Recently, it has been shown that in addition to the localization in the plasma membrane and endoplasmatic reticulum (Anandatheerthavarada 2003), APP may be targeted to mitochondria. The accumulation of APP in the mitochondrial compartment caused mitochondrial dysfunction and impaired energy metabolism in cortical neuronal cells. Thus, it is possible that due to the overexpression of APP, more APP is also localized in mitochondria of APP transfected PC12 cells, leading to the observed impairment energy metabolism. In fact, accumulation of APP in mitochondrial fraction could be detected by western blotting in both APPwt and APPsw PC12 cells (Keil 2004). The results are reduced ATP levels and reduced metabolic activity as detected by the MTT assay (see. 3.3.3) in APPsw and APPwt compared to vector control cells. The accumulation of overexpressed APP in mitochondria might explain the differences in ATP levels and metabolic activity between APPsw and APPwt, since enhanced expression of human APP has been found in mitochondria of APPsw transfected PC12 cells (Bonert 2004).

Relevance of nitric oxide (NO) and mitochondrial failure in beta amyloid-induced cell death?

In addition to our results, several studies have also observed activity changes in mitochondrial enzymes in AD brain including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Gibson 1998). An impaired cytochrome c oxidase activity has further been found in the CNS and tissues including platelets of AD patients (Maurer 2000; Cardoso 2004). Moreover, accumulating evidence suggests that in addition to oxidative stress, also nitrosative stress is an early events in the pathogenesis of AD (De la Monte 2003; Law 2002; Jang 2004). Nitric oxide (NO) and its derivates (RNS) are known to inhibit the mitochondrial respiration (Brown 2002). NO itself causes a selective and reversible inhibition of cytochrome c oxidase, whereas RNS inactivate multiple respiratory chain complexes (I, II , III, IV) and ATP synthase (Radi 2002).
Experiments from Uta Keil revealed that overexpression of APPsw increased the NO levels in PC12 cells and HEK cells. In addition, the treatment of PC12 cells with extracellular $Aß_{1-42}$ also leads to an increase of NO levels but to a lesser extent than in APPsw PC12 cells. Thus, it can be hypothesized that the enhanced intracellular Aß production in APPwt and APPsw cells is responsible for the Aß-dose dependent NO increase (Keil 2004). Hence, it is probable that the mitochondrial dysfunction is induced by the enhanced NO levels, or by a direct effect of Aß on mitochondrial function, inhibiting key metabolic enzymes and contributing to the deficiency of energy metabolism seen in AD.

Impact of the beta-amyloid- induced mitochondrial dysfunction

The consequences of Aß-induced mitochondrial dysfunction are obvious. Under basal conditions, the chronic low-levels of intracellular Aß (picomolar range) in APPsw PC12 cells leads to reduced ATP levels and reduced metabolic activity. Nevertheless, the cells seems to be adapted to this energy deficiency, since no enhanced basal apoptotic levels has been measured. If this adaptation occurs through a upregulation of glycolysis needs to be determinated e.g. by measuring the activation of the energy charge-sensitive AMP-activated protein kinase or the 6-phosphofructo-1-kinase, the master regulator of glycolysis.
In addition, proteins of the Bcl-2 family, which are important regulators of apoptosis are also affected, because the Bcl-xl/Bax ratio is reduced in APPsw cells (Keil 2004). This shift in the Bcl-xl/Bax ratio towards the proapoptotic Bax, and the energy deficiency conduct APPsw cell

to be more vulnerable to mitochondrial membrane potential changes and ATP reduction after oxidative stress. The consequence is an activation of the intrinsic apoptotic pathway, releasing cytochrome c into the cytosol, resulting in an activation of the apoptotic protease-activating factor-1/caspase 9 apoptosome complex.

Since we determined no differences in cytochrome C release and caspase 9 activation between APPsw and APPwt cells other effects of the altered Bcl-xl/Bax ratio and energy deficiency need to be considered. Interestingly, experiments performed investigating AIF, which induced apoptosis independent of caspases, show that AIF protein expression is enhanced in mitochondria when oxidative stress is induced. This enhanced AIF expression was gene-dosis dependent in following order: APPsw > APPwt > vector control. In addition, another mitochondrial apoptotic factor, the Smac protein was analyzed by Astrid Bonert. The release was enhanced under basal conditions and after oxidative stress in APPsw and APPwt cells compared to vector transfected control cells which is in agreement with this obtained caspase 9 results.

Our results were confirmed by a very recent publication (Katoh 2004). Using U937 cells, hydrogen peroxide caused a subtle decrease in the inner membrane potential with cytochrome c release and caspase 9 activation, whereas Bcl-2 overexpression cause render cells resistant to oxidative stress.

4.3 THE IMPLICATION OF THE JNK ACTIVATION IN NEUROTOXIC MECHANISM INDUCED BY THE SWEDISH APP MUTATION

Mitogen-activated protein (MAP) kinase signaling pathways relay, amplify and integrate signals from a diverse range of extracellular stimuli, thereby controlling the genomic and physiological response of a cell to changes in the environment. These responses include cellular proliferation, differentiation, development, the inflammatory response and apoptosis. In neuronal cells, potentially deleterious stimuli, such as deprivation of trophic factors, UV irradiation, free radicals, hypoxia, ischemia, heat shock, and cytokines, provoke an intracellular stress response that either leads to apoptosis or defensive-protective adaptations. The central mediator thereby is SAPK (stress activated protein kinase) also designated as c-Jun NH2-terminal kinase (JNK). The other described stress-activated MAP kinase is the 38

kDa protein kinase p38, which is activated by bacterial endotoxins, inflammatory cytokines and osmotic stress (Mielke 2000).

JNK is a serine/threonine kinase and activated through multiple protein kinases by dual phosphorylation at conserved threonine and tyrosine residues, whereas the phoshorylation of Thr183 and Tyr185 is essential for the kinase activity. Activated JNK in turn is known to phosphorylate and activate several transcription factors like c-Jun at Ser63/73. Due to alternative splicing, ten JNK isoforms exist yielding proteins of 46 and 55 kDa.

An activation of the JNK pathway in cortical neurons exposed to Aß was reported recently by Morishima et al. (2002). The phosphorylation and activation of the c-Jun transcription factor by JNK was shown to stimulate the transcription of several key target genes, including the death inducer Fas ligand. The binding of Fas ligand to its receptor Fas then induces a cascade of events that lead to caspase activation and ultimately cell death. In addition, in other reports the activation of JNK associated with amyloid deposition was observed in mice transgenic for human APPsw mutation (Savage 2002).
A direct link between oxidative stress and JNK has been shown by the demonstration that JNK is activated by 4-hydroxynonenal (HNE), a major aldehydic end product of lipid peroxidation (Parola 1998; Uschida 1999).

Given the important role that JNK pathway plays in cellular stress signalling, we investigated the relationship between activated JNK, apoptosis and the Swedish APP mutation. We demonstrated that oxidative stress leads to an activation of JNK. JNK phosphorylates specific sites in the N-terminal region of c-Jun, thereby enhancing its transcriptional activity. Very importantly, we were able to demonstrate that in PC12 cells bearing mutant APPsw the activation of JNK and c-Jun was significantly enhanced compared to APPwt cells. At the same time activation of JNK and c-Jun is increased in APPwt compared to vector cells.

Swedish APP mutation induced activation of JNK

The obtained results show that in oxidative stress induced cell death the Swedish APP mutation led to an enhanced activation of JNK in PC12 cells. This suggests that the different Aß levels in the PC12 cell lines induce a dose-dependent activation of JNK pathway. At this point several questions are arise: How is JNK activated in oxidative-stress induced cell death? And why is the JNK activation enhanced by the Swedish APP mutation?

As described above, the energy status of the cell, and here especially the ATP concentration, plays a crucial role in cell survival. Prolonged ATP depletion leads to cell death. Small changes in ATP levels do not necessarily result in cell death but are sensed by cells. A fall in ATP with a subsequent rise in AMP activate the AMPactivated protein kinase (AMPK). AMPK acts as a metabolic master switch by inactivating key enzymes of anabolic processes that consume ATP. It also favours fattyacid oxidation and, in certain cells, it stimulates glycolysis by increasing glucose transport and by activating 6-phosphofructo-2 kinase thereby favouring ATP production. ATP conservation is therefore the net result of AMPK activation, which contributes to liver cell survival in the short term (Hue 2003). However, consequences of prolonged stress and of a sustained AMPK activation triggers cells to apoptosis by the activation of JNK as it has been shown recently (Kefas 2003; Larsen 2002). Measurements by Uta Keil indicate that after a 30 min hydrogen peroxide incubation ATP levels in PC12 cells falls down strongly. In addition, the recovery of the ATP levels is delayed and weaker in APPsw PC12 cells. Thus, it is probably that the JNK activation in oxidative stress is catalysed by the AMPK.

In addition to AMPK, there is accumulating evidence that JNK activation is associated with TNF- and Trail receptor-mediated apoptosis (Arch 1998; Vivo 2003; Reveneau 2003; Deng 2003; Schwabe 2004). Thereby JNK is activated by ASK1 (Apoptosis signal-regulating kinase 1), which is known to bind a death receptor associated adapter proteins TRAF2 through direct protein-protein interaction. ASK1 itself is a mitogen-activated protein kinase kinase kinase (MAPKKK) family member upstream of JNK. Very recently an activation of JNK by hydrogen peroxide has also been reported to occur via TNF receptor (Pantano 2003). In fibroblasts from TNF-receptor-deficient mice the ability of hydrogen peroxide to activate JNK was inhibited compared with fibroblasts from control mice. Simultaneously,

immunoprecipitation of TNF-R1 revealed that in response to hydrogen peroxide the adapter protein, TRADD and JNK were recruited to the receptor. However, these observed activation of JNK in TNF- or Trail receptor apoptosis has never been observed in neuronal cells, and the implication of these death receptors for the role of JNK in Aß toxicity needs to be further classified.

In addition to the report implicating an APP-dimer complex formation for the activation of caspase 8. Recently, Hashimoto et al. (2003) reported a complex formation of dimerized APP with ASK1 and JNK via the scaffold protein JIP-1b (JNK interacting protein). JIP family proteins are scaffold proteins that organize specific members of the JNK/MAPK cascade to facilitate signalling. Fusion constructs of the epidermal growth factor (EGF) receptor with cytoplasmic domain of APP were expressed in neurohybrid cells, and here the addition of EGF enhanced neuronal cell death in a caspase 3 dependent manner. Apoptosis was inhibited by a dominant-negative ASK1 and SP600125. JIP-1b and ASK1 co-immunoprecipitated with APP, but not with APP lacking the YENPTY-motif. Thus, it was proposed that APP/JIP-1b/ASK1 form a ternary complex. Indeed, immunoprecipitation were only obtained when each molecule was overexpressed. Whether endogenous proteins are also able to form such a ternary complex needs to be verified.

Very interestingly are reports proposing that the JIP1b scaffold protein could serve as cargo for the microtubule motor kinesin to mediate the transportation of several transmembrane proteins (Taru 2002b) in which APP is supposed to be a cargo receptor (Kamal 2001). In addition, JNK has been implicated in the modulation of the APP metabolism, since it phosphorylates APP within the carboxy-terminus of APP at Thr 668. This phosphorylation is suggested to play an important role in controlling the metabolism and physiological functioning of APP (Sisodia 2002). Since cellular stress (inhibition of protein synthesis by anisomycin or UV radiation) leads to a phosphorylation of APP by JNK (Taru 2004), and the Aß production is significantly reduced when phosphorylation of T668 is abolished by mutation (Lee 2003, it is further assumable that phosphorylation of APP may increase Aß generation.

Our data clearly indicate that the altered processing of APPsw might play a crucial role in activation of stress kinase pathway. If this occurs by a redistribution of JIP scaffold proteins or by altering APP metabolism has to be clarified in the future.

4.3.1 JNK inhibitor protects mitochondrial membrane potential and reduces caspase 9 and 3 activity

Interestingly, using the selective JNK-inhibitor SP600125, a reduction of caspase 9 activity was observed whereas activities of caspase 2 and caspase 8 were unaltered. Furthermore, JNK translocates to mitochondria, and the JNK inhibitor compensated the mitochondrial abnormality when oxidative stress was induced. Due to the fact that members of the anti-apoptotic Bcl-2 f protein family are inactivated through phosphorylation by JNK (Inoshita 2002; Maundrell 1997) our results support evidence that JNK alters mitochondrial function. Furthermore, the Western blots of the mitochondrial fraction show that JNK translocates to mitochondria during oxidative stress.

Our results have been confirmed by other recent reports: JNK was found to be strongly activated in APP transgenic mice with extensive oxidative damage, but not in APP transgenic mice with little oxidative damage (Zhu 2001; Hwang 2004). In Jurkat cells JNK was activated by ROS and caspase 2 and -9 processing as well as cytochrome c release inhibited by SP600125 (Dirsch 2004). Furthermore, SP600125 delayed the mitochondrial permeability transition, inhibited cytochrome c release and prevented Bid degradation in TNF-alpha induced apoptotsis in mouse hepatocytes (Schwabe 2004). Investigations by Isabel Scherping from our lab revealed a protective effect of SP600125 on mitochondrial membrane potential of dissociated mice neurons incubated with Aß, also suggesting that JNK-regulated proapoptotic factors act upstream of the mitochondria.

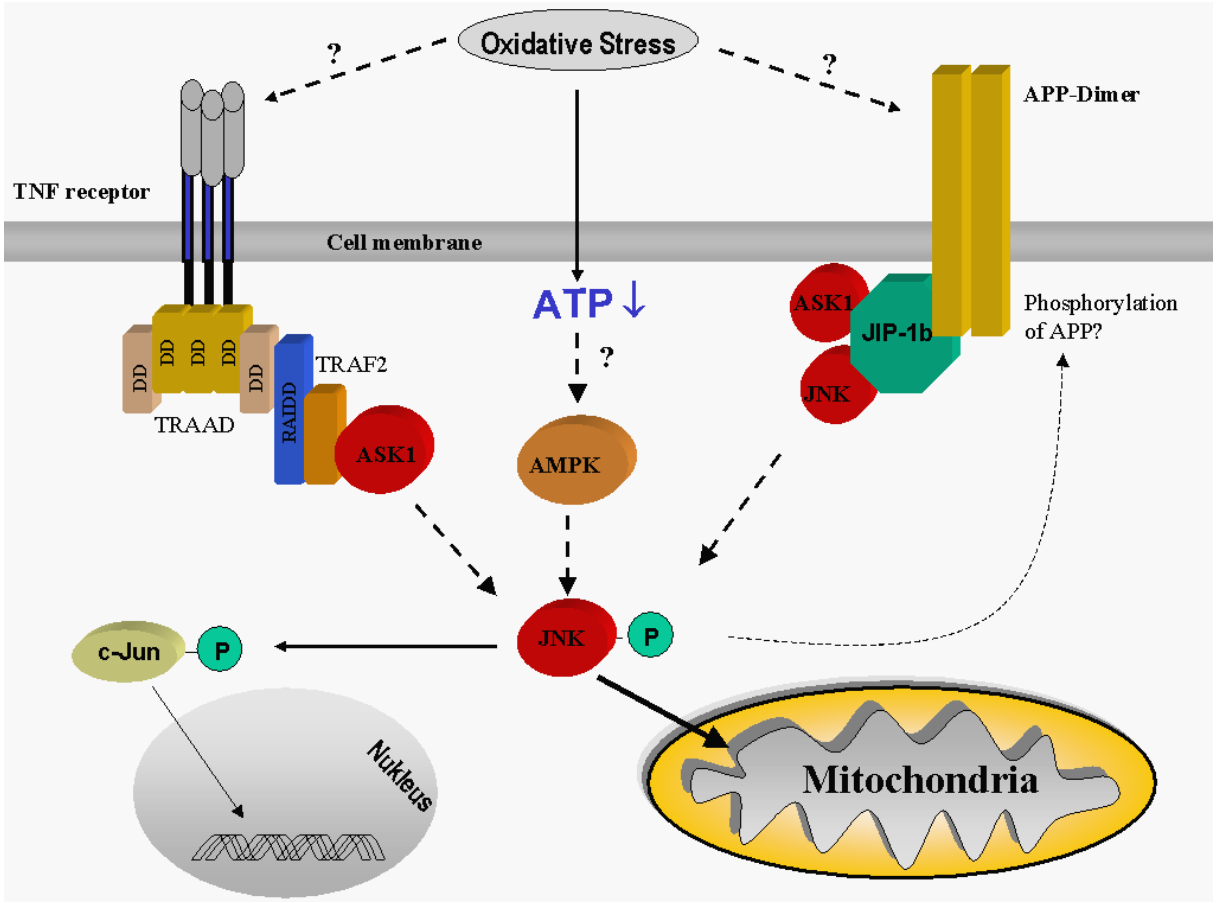

Fig. 4.1 Possible mechanisms for activation of JNK in PC12 cells. Since both, APPsw and APPwt transfected PC12 cells have APP overexpression, an enhanced activation of JNK by the formation of an APPdimer,JIP1b, ASK1 and JNK-complex is assumable. Due to reduced ATP levels in APPsw cells the activation of AMPK could result in the phosphorylation and activation of JNK.

4.4 EFFECTS OF CASPASE INHIBITORS ON OXIDATIVE STRESS INDUCED CELL DEATH IN PC12 CELLS

In addition to determination of caspase expression and activity, the importance of caspases were investigated using specific caspase inhibitors. The protective effects of caspase inhibitors were analysed using different methods. Apoptotic cell death was measured using the DNA dye propidium iodide and FACS analysis, plasma membrane leakage was detected with trypan blue dye and metabolic activity was examined by the reduction of cellular 3-(4,5-dimethlythiazol-2yl)-2,5-diphenyltetrazolium to formazan. Measuring apoptotic cell death the results were surprising, since only the caspase 3 inhibitor was able to reduce oxidative stress-induced apoptosis

significantly in APPsw and APPwt transfected PC12 cells. The inhibition of caspase 2, caspase 8 and caspase 9 was less effective in preventing apoptosis, although the inhibitors show efficiency in reducing the respective activities in lysates of treated cells. The lack of efficacy of the initial caspase inhibitors to abolish apoptotic cell death could be completely attributed to the further consisting compensatory cell death pathway. Although the treatment of PC12 cells with hydrogen peroxide leads to activation of these initiator caspases, their inhibition is not enough to avoid apoptosis, because different caspase pathways co-exist (Fig. 3.2). Such a compensation of caspase pathway was ascertained in sympathetic neurons from caspase 2 null animals. A lack in caspase 2 expression was compensated by an increased expression of both mRNA and protein for caspase 9 and DIABLO/Smac in trophic factor deprivation-mediated death (Troy 2001).

The failure of the caspase inhibitor mix (containing caspase 3, caspase 8 and caspase 9, 10 µM each) in preventing oxidative stress induced apoptosis in PC12 cells could be a result of the toxic effects of caspase inhibitors at high concentrations. As determined by pre-experiments, the addition of 50 µM caspase 3 inhibitor enhanced apopototic rates of PC12 cells compared to controls. Protective effects were only measured using 1µM or 10 µM caspase 3 inhibitor. Here the mix containing a total concentration of 30 µM may also be toxic. Thus, experiments mixing caspase inhibitors at lower concentrations could result in a better protective effects, which however needs to be tested.
As detected with trypan blue staining, the incubation with increasing concentrations of hydrogen peroxide leads to increased rates of PC12 cells showing membrane leakage, whereas at high concentrations (up 500 µM) a shift from apoptosis towards necrotic cell death occurs, because the rate of membrane leakaged cells (which occurs in both apoptosis and necrosis) increased, while the apoptotic rate declined. As determined by trypan blue staining, no significant reduction of caspase inhibitors in preventing absolute cell death could be ascertained.

APPsw PC12 cells show reduced metabolic activity

The MTT assay is a widespread used assay to measure cytotoxic effects in cell culture. It allows to measure the metabolic activity, quantifying the reduction of -(4,5-dimethlythiazol-2yl)-2,5-diphenyltetrazolium to formazan. This reduction is catalysed by redox enzymes including enzymes of the respiratory chain like succinatdehydrogenase but it has also been shown that

cellular MTT reduction occurs outside the mitochondrial inner membrane and involves NADH and NADPH-dependent mechanisms that are insensitive to respiratory chain inhibitors (Berridge 1993).

Parallel to the lower ATP level in APPsw PC12 cells, the metabolic activity was also found to be reduced. The incubation with hydrogen peroxide reduce metabolic activity in a concentration dependent manner. While the pre-treatment of caspase inhibitors at a 100 µM hydrogen incubation show no or only a weak effects, at higher concentrations (250 µM) a protective effects was measured by caspase 3, caspase 8 as well as caspase 9 inhibitor. Thus, it seems that caspase inhibitors lead to some protection of the cells, since the metabolic activity was higher as in cells with no caspase inhibitor pre-treatment.
However, the results from the propidium iodide staining and FACS analysis show no significant protection by caspase 8, caspase 9 or the caspase inhibitor mix, which may suggest that despite a samll protection of the metabolic acticity, the DNA of hydrogen peroxide treated PC12 is fragmented, since an enhanced sub G1 peak is detected. In addition, it will be interesting to elucidate how much of the metabolic activity is required for the execution of apoptosis, because it has been shown that apoptosis is an active process that requires a complex of tightly regulated ATP-dependent steps. Indeed, ATP is required to switch on genes that are involved in the early phases of apoptosis caspases (Thornberry 1998). Here, it has been shown that a decrease in ATP levels below to 20% of basal ATP leads to a shift from apoptosis to necrosis, whereas these type of cell death has been determined “aponecrosis” (Formigli 2000).

These results show that the widespread and easy to handling MTT assay should not be the only method used to measure cytotoxicity and that the differences in the detection methods should be concerned in comparing various results.

4.5 BASAL APOPTOSIS IS INCREASED IN APPSW HEK CELLS

HEK cells overexpressing APP show increased basal apoptotic levels compared to untransfected control cells. This is in contrast to the measured apoptotic levels of PC12 cells, where in the absence of apoptosis-inducing treatments, no evidence has been found for increased apoptosis in APPsw transfected PC12 cells compared to APPwt cells or control

cells. Calculating the Aß production in the two cell lines transfected with the Swedish APP mutation, the HEK cells have a nearly 30-fold higher Aß level than the PC12 cells (HEK APPsw 5.42 nM; PC12 APPsw 0,20 nM). Thus, using the different cell lines allow to study dose-dependent effects of Aß, and it is attempting to hypothesized that the 30-fold higher Aß levels leads to the enhanced basal apoptosis in HEK cells.
In parallel experiments performed in our lab, treating PC12 cells extracellularly with $Aß_{1-42}$ at a concentration of 100 nM and higher, results also in increased apoptotic cell death (Keil 2004). Interestingly, this effect on the induction of apoptosis was less pronounced in the extracellular setting than in APPsw HEK cells, showing that the cells are more vulnerable to high chronic Aß stress than to the extracellular addition of synthetic $Aß_{1-42}$ peptides.
However, no significant differences in basal apoptotic rates between APPsw and APPwt HEK cells could be detected, although APPsw transfected HEK cells have a tenfold higher Aß production as APPwt HEK cells. This means that either the lower Aß levels from APPwt HEK cells were already sufficient to prime HEK cells to enhanced basal apoptosis, or that other effects needs to be considered, like accumulated APP in mitochondria.

4.5.1 The Swedish APP mutation impairs metabolism in HEK cells

Similar as in PC12 cells, ATP levels were significantly decreased in HEK cell transfected with the Swedish APP mutations compared to APPwt or control. In addition, ATP levels in APPwt HEK cells were lower than the control. Thus, it is assumable that the increasing Aß production from APPwt to APPsw results in a dose-dependent decline in ATP levels, whereas the effect is stronger in HEK cells as in PC12 cells.
In accordance to ATP levels, a reduced mitochondrial membrane potential was ascertained for HEK cells in the following order: APPsw < APPwt < control, whereas APPsw bearing PC12 cells showed a slightly hyperpolarized mitochondrial membrane potential compared to APPwt or vector control cells.

Aß impairs energy metabolism

The obtained results show clearly that Aß seems to impair the energy metabolism of mitochondria. This conclusion is also supported by other experiments from our lab. In 3-month-old APP tg mice reduced ATP levels were observed under basal conditions compared to littermate control mice, showing the *in vivo* relevance of the cell culture findings

(Scherping 2004). Interestingly, 3 month-old mice exhibit no plaques but detectable Aß levels in the brain suggesting the very important role of intracellular Aß in ATP reduction, which support the hypothesis that intracellular Aß affects cellular functions and may represent the primary event within the neurotoxic Aß cascade. In older, 12-month-old amyloid plaque-bearing transgenic mice bearing the Swedish APP mutation reduced COX activity and reduced ATP levels were also found (Anandatheerthavarada 2003). In addition, it was shown, that impaired brain metabolism always occurs in clinically significant AD, and the degree of clinical disability is proportional to the degree of metabolic impairment (Blass 2002). Since the earliest, mildest changes in brain metabolism occur even before the onset of measurable cognitive impairment or atrophy (Hoyer 2004), this also pointed for the primary event of intracellular Aß for the development of the disease.

4.5.2 Caspase inhibition in HEK cells

As discussed above, the HEK cells transfected with APPwt or APPsw have enhanced basal apoptotic levels in comparison to untransfected control cells. This is probably due the enhanced Aß production, which increase NO affecting respiratory chain. Nevertheless, also a direct effect of accumulated APP on mitochondria or other effects cannot be excluded, because there is no significant difference in basal apoptotic rates between HEK APPwt and HEK APPsw, though APPsw HEK have an nearly 10 fold production of Aß as APPwt. To elucidate the implications of caspases, HEK cells were treated for 24 hr with several caspase inhibitors at a final concentration of 10 µM. Here the measured effects were moderate. Only caspase 2 inhibitor was able to show a significantly protective effect in reducing basal apoptosis in APPsw HEK cells. This point towards a pivotal role of caspase 2 in Aß induced apoptosis as described in 4.2.1 also for HEK cells. However, the failure of caspase 2 inhibitor to protect mitochondrial membrane potential in oxidative stress induced cell death show that in addition to acting upstream of mitochondria, caspase 2 might induce apoptosis in a distinct way, e.g. a non-mitochondrial apoptotic pathway.

It has also to be mentioned, that due to a manner of time, it was not measured if caspase 2 inhibitor may elevated basal membrane potential of HEK APPsw and HEK APPwt to levels of untransfected control cells. This experiment may be essential to elucidate if caspase 2 has an effect on mitochondria in HEK cells. In addition, it will be necessary to investigated the

implication of caspases by measuring caspase activities and performing Western blots to obtain a more precise conclusion, according to the implication of caspases in PC12 cells.

4.5.3 JNK activation in HEK cells

Similar to PC12 cells, an induction of JNK activation by the Swedish APP mutation was also found in HEK cells. Oxidative stress induced JNK activation in a time depend manner, whereas in APPsw cells JNK was found to be activated even under basal conditions. In addition, JNK was also detected in the mitochondrial fraction, which may suggest that JNK act upstream of mitochondria in oxidative stress induced apoptosis. However, experiments pre-adding JNK inhibitor prior the hydrogen peroxide incubation show no protective effect of JNK in preventing the oxidative stress induced decrease of the mitochondrial membrane potential, as it was measured in PC12 cells. This could be due following reasons:

- Other nonmitochondrial effects like the upregulation of apoptotic genes by JNK pathway (Morishima 2002) are further implicated in HEK cells.
- The higher Aß load in HEK cells counteracts the protective effect of JNK inhibitor by damaging mitochondria independent of JNK.

4.6 SWEDISH APP MUTATION INDUCES TRANCRIPTION OF AIF

As it has been described, in addition to their vital role as the cellular power stations, mitochondria exert an important function in apoptosis. In response to oxidative stress mitochondrial membranes are permeabilized, leading to the release of mitochondrial proteins. Such pro-apoptotic proteins include cytochrome c, which can trigger the activation of caspases, once it has reached the cytosol as well as the caspase-independent death effector AIF (Apoptosis inducing factor). AIF is a mitochondrial flavoprotein which, in healthy cells, is confined to the mitochondrial intermembrane space and has an NADH oxidase activity independent from its apoptogenic effects. Translocation of AIF from the mitochondria to the nucleus has been shown to promote apoptosis (Susin 1999) and seems to play a crucial role during caspase-independent apoptotic cell death (Bidere 2001). When released from the mitochondria, AIF acts as a death effector, migrating to the nucleus and inducing chromatin condensation and large-scale DNA fragmentation in a caspase independent manner (Daugas 2000).

Here the RT-PCR data clearly show that oxidative stress upregulates the level of AIF mRNA in HEK cells, suggesting that the modulation of gene transcription and/or mRNA processing or stability of mRNA are involved in this effect of hydrogen peroxide. In addition, an upregulation of AIF mRNA was also observed in PC12 cells. Upregulation of AIF expression in HEK and PC12 cells treated with hydrogen peroxide may be due to the augmentation of gene transcription changes in processing or the stability of mRNA. Interestingly, this effect was cleary enhanced in HEK cells transfected with the Swedish mutated APP. In PC12 cells mRNA expression was little enhanced after 1 hr induction in both, APPsw and APPwt compared to vector control cells. Experiments performed by Astrid Bonert revealed an higher AIF protein expression in APPsw cells after 6 hr hydrogen peroxide incubation. Thereby, AIF was not found in cytosolic fraction in the first 6 hr, but after 24 hr incubation, suggesting an important role of AIF in the late phase of apoptosis. In further experiments it will be interesting to find out, how AIF expression is induced by the Swedish APP. Possible is a direct effect of Aß. This could be verified for example using γ-secretase inhibitors, reducing Aß levels of APPsw transfected cells.

It is also possible that the C-terminal fragment of APP (AICD) could be responsible for an enhanced AIF expression, since AICD has been demonstrated to cause transcriptional activation after translocalization into the nucleus hence suggesting a role of AICD in gene regulation (Kinoshita 2002, Kim 2003).
The implication of AIF could be an explanation for the failure of caspase inhibitors to reduce apoptotic levels more distinctly. Especially in HEK cells caspase inhibitors fail to inhibit oxidative stress induced cell death more clearly. In fact, caspase 3 inhibitor reduces apoptotic levels significantly, but the observed effect was far from reducing the levels to apoptotic rates of hydrogen peroxide incubated untransfected HEK cells. In further experiments it will be also interesting to combine caspase inhibitors with drugs, that may prevent release of AIF.

4.7 NEUROTOXIC MECHANISMS CAUSED BY THE SWEDISH APP MUTATION: MITOCHONDRIAL DYSFUNCTION, OXIDATIVE STRESS, CASPASES AND JNK PATHWAY

Summarizing all the obtained result it becomes evident that different pathways contribute to the enhanced vulnerability of cells harbouring the Swedish APP mutation. While probably high chronic Aß levels lead directly to enhanced apoptotic levels, reduction in mitochondrial membrane potential and depletion of ATP levels in APPsw transfected HEK cells, the chronic lower levels of Aß only trigger APPsw PC12 cells to enhanced apoptosis after a secondary insult.

When oxidative stress is induced several pathways may occur in APP-transfected PC12 cells in parallel. The intrinsic pathway is activated in APPwt and APPsw bearing cells leading to mitochondrial dysfunction in an Aß-dose-dependent manner compared to vector-transfected controls. Mitochondria may act as amplifiers, because cytochrome c is released and activates caspase 9. Since no differences in cytochrome c release and caspase 9 activity between APPsw and APPwt cells were observed, other pro-apoptotic factors from the mitochondria like AIF might be involved since AIF expression is enhanced by the Swedish mutation.

The 4–5-fold elevated Aß levels in APPsw compared with APPwt cells resulted in enhanced JNK, caspase 2, and caspase 8 activation. Here a connection to the intrinsic apoptotic pathway could exist, since the inhibition of JNK and caspase 2 resulted in a protective effect on mitochondrial membran. The convergence of all pathways leads to activation of the caspase 3 and the execution of cell death. Thus, the blocking of initiator caspases only inhibits the amplification loop and does not prevent cell death. If the enhanced AIF expression is due an effect of caspase 2, JNK and enhanced transcriptional activity of c-jun, AICD or due to another effect needs to be further investigated.

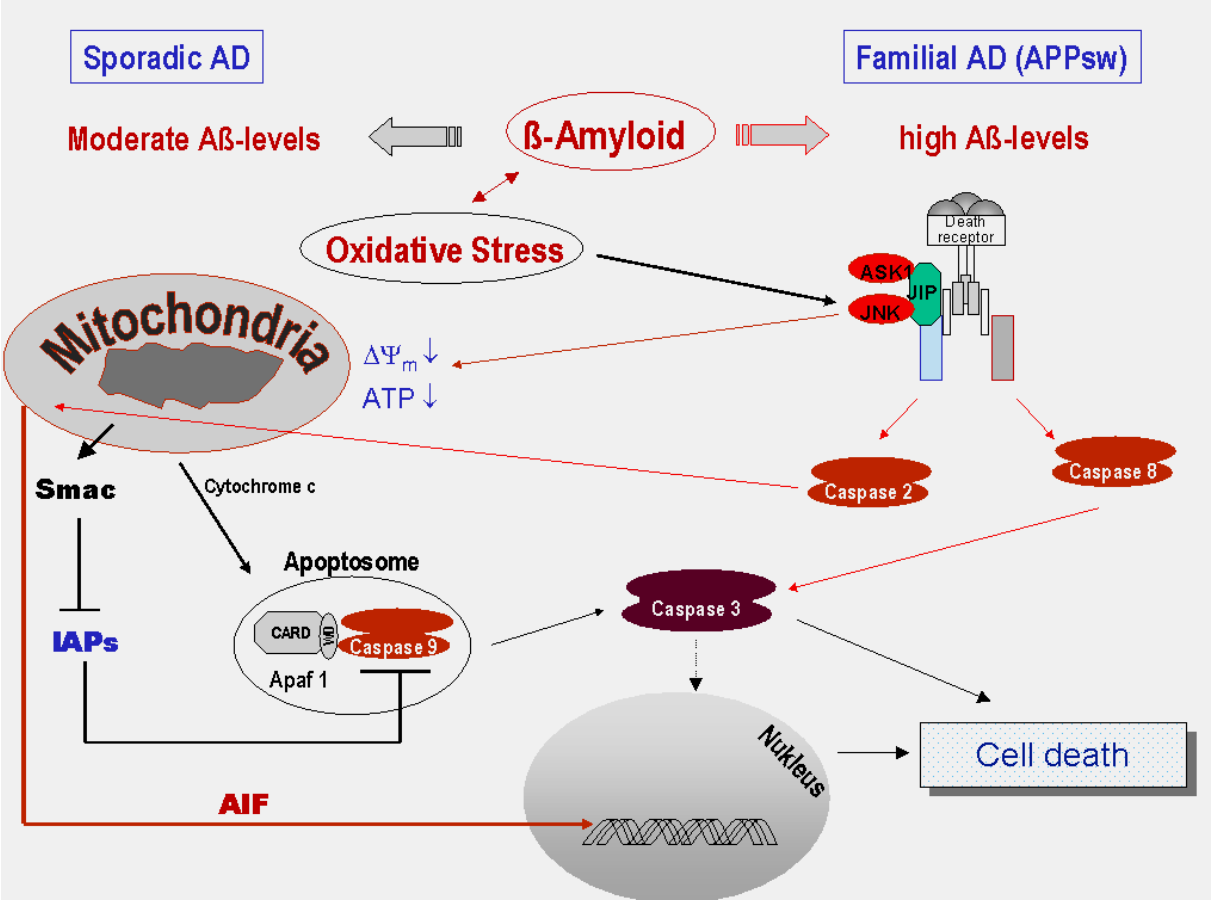

Fig. 4.2 Hypothetical sequence of events leading to cell death occurring in FAD. Effects of the Swedish APP mutation are indicate by the red arrows.

Based on this thesis it can be speculated that these upregulated pathways are also responsible for the enhanced vulnerability of neurons of Swedish FAD patients. The consequence of the gradually rising in oxidative stress throughout life is a massive neurodegeneration leading to a start of the disease below an age of 65. In sporadic AD cases Aß levels gradually arises with age, here rising levels of oxidative stress lead to neuronal cell death occurs only over an age of 65. Since other FAD mutations also emphasis Aß production, it is assumable that the same pathways may be upregulated, which, however, needs to be confirmed.

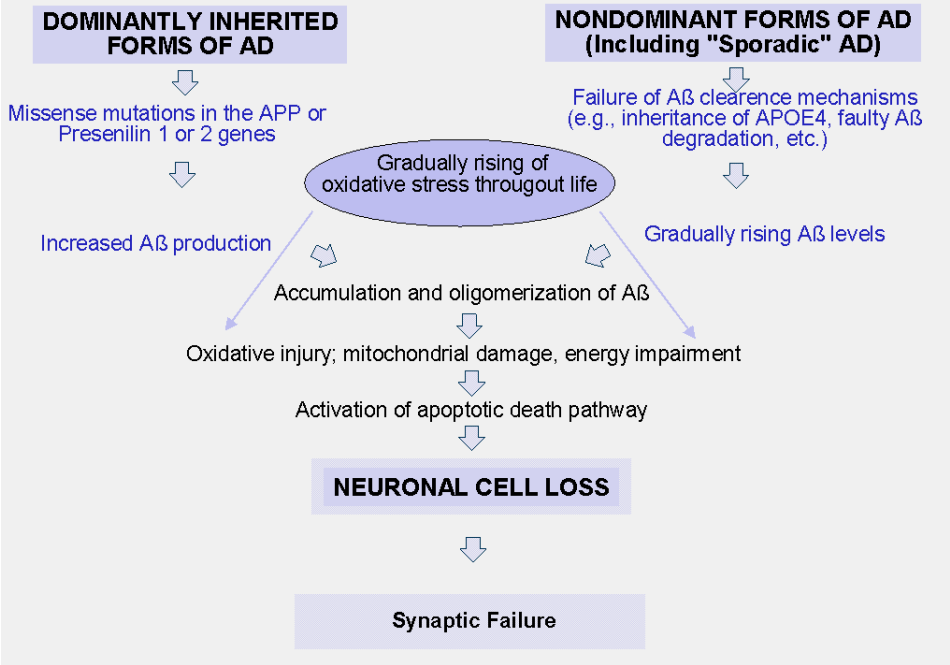

Fig. 4.3 A hypothetical sequence of the pathogenic steps linking sporadic AD, familiar AD, and Aß production with mitochondrial dysfunction, apoptotic pathways and neuronal cell loss (modified according to Selkoe 2002). Due to enhanced Aß production, neuronal cells of FAD patients are more vulnerable to the gradually rising of oxidative stress throughout life, leading to an early onset form of the disease. The deposition of Aß in senile plaques is a final step leading to inflammatory response and dementia.

4.8 NEUROPROTECTIVE STRATEGIES INHIBITING CASPASES AND JNK

As this thesis shows, inappropriate apoptosis clearly underlies the etiology of AD, In addition altered apoptosis has also been implicated in several other human diseases like cancer, autoimmunity or heart disease (Hetts 1998). The control of caspases, as a key and central component of the biochemical pathway that mediates apoptotic cell death, represents an attractive step in modulating this process. There are efforts to induce caspase activation for the treatment of disorders where insufficient apoptosis occurs (e.g. cancer). Also in HIV infection efforts to activate caspases in infected cells exist; e.g. a “Trojan horse” gene therapy approach may be viable, in which a TAT-caspase-3 construct containing a HIV-protease recognition motif selectively induces apoptosis in HIV infected cells only (Vocero 1999).

Alternatively, selective caspase inhibition for the treatment of AD comprised a big challenge. The clinical utility of caspase inhibitors will depend on several key issues that require further resolution. For example, will cells that are saved from apoptotic death remain functional and survive without perpetual caspase inhibition? Will the "therapeutical window" be sufficient for practical use in a clinical setting? Can highly selective caspase-3 inhibitors be used for chronic administration without unacceptable adverse events occurring?
In other neurodegenerative diseases there are encouraging approaches. Preliminary experiments in animal models using non-selective caspase inhibitors such as Z-VAD(OMe)-CH2F, have shown *in vivo* efficacy in ischemic and hypoxic brain injury (Cheng 1998; Daemen 1999).

In another neurodegenerative disorders, Huntington's disease, the use of minocycline as an inhibitor of apoptosis delayed mortality of transgenic mice (Chen 2000). Minocycline is a second-generation tetracycline with remarkable neuroprotective properties. It inhibits the production of nitric oxide by the inducible form of nitric oxide synthase, and was evaluated in experimental models of cerebral ischemia. Currently it is being evaluated in clinical trials in patients with Huntington's disease (Bonelli 2004). An advantage is that Minocycline is orally bioavailable, crosses the blood–brain barrier, and has a proven safety record in humans. Whether Minocycline might be considered for trials in AD still needs to be tested.

An alternative approach to neuroprotection will be the inhibition of JNK, since JNK activation seems to be a critical event in the primary neurotoxic signaling triggered by Aß. In AD brain JNK activation has also been found by immunostaining (Shoji 2000, Zhu 2001) including in association with intraneuronal Aß accumulation and in association with tangle-like inclusions in entorhinal cortex before Aß deposition (Pei 2001, Savage 2002). Interestingly, evidence suggests that JNK activation also contributes to tau phosphorylation. Since JNK inhibition blocks caspase activation, the JNK inhibition is an assumable trial for AD therapy. In addition to SP600125, another compound that inhibits stress activated kinase signalling is CEP-1347, which is an inhibitor of the MLK family of JNK pathway activators (Harris 2002). CEP-1347 is currently undergoing clinical trials in Parkinson's disease and is a candidate agent for AD trials (Parkinson Study Group, Neurology 2004).

4.8 IMPLICATION OF PRESENILIN 1 IN APOPTOSIS

As it was described in 1.3, most of the aggressive, early-onset forms of familial AD are caused by mutations in the PS genes. PS1 and PS2 are homologous, polytypic membrane proteins localized to ER and Golgi membranes. PS1 and PS2 span these membranes six or eight times, and most of the mutations identified in AD families cluster within or adjacent to the transmembrane domains (Haass 2002). Between the putative sixth and seventh transmembrane domain, the PSs contain a hydrophilic, acidically charged loop that contains proteolytic cleavage sites. Three different proteolytic systems, including caspase cleavage, are known to be involved in PS catabolism. PSs normally undergo endoproteolytic cleavage in a hydrophobic portion of the cytoplasmic loop, which generates stable N-terminal and C-terminal fragments that associate in a 1:1 ratio. PSs are also cleaved by caspase 3 within the cytoplasmic loop at positions distal to the endoproteolytic site. For example, PS1 is cleaved at D345 and PS2 has a major caspase-3 site at D329 and a minor caspase-3 site at D326 (Kim 1997; Vito 1997). The discovery of PSs as caspase substrates raised the interesting possibility that they may play an important role in the apoptotic death.

4.8.1 Presenilin 1 overexpression reduces oxidative stress induced apoptosis in PC12 cells

PS1 is critically involved in γ-secretase activity and might represent the protease that cleaves APP within its transmembrane domain, finally generating Aß. Since this thesis show that Aß clearly enhance apoptosis, it first seems to be paradox that an overexpression of PS1 in PC12 cells may have a protective effect in oxidative stress induced apoptosis. The explanation however, is obvious:

- PS1 assembles to a high molecular weight complex with other proteins including Nicastrin, Aph-1 and Pen-2 that are essential for γ-secretase activity. Since taking away a single of these components disrupt the entire γ-secretase activity, here the overexpression of one of these components alone might not increase the generation of Aß.
- In addition, the physiological role of PS1 is not clear. The γ-secretase activity is also essential for Notch signalling (see.1.4.3), but PS1 might not only be involved in intermembraneous proteolysis, since it has also been implicated in the regulation of calcium homeostasis (Mattson 1997; Yoo 2000, Leissring 2000) and in the regulation of Wnt pathway (Xia 2001; Kang 2002).

Thus, it is rather assumable, that the over 120 PS1 related FAD mutations induce an imbalance between a loss of physiological function and proteolytic processing of APP. A direct effect of the mutations on the proteolytic activity may be improbably.

One possible physiological function of PS1 might be implicated in the regulation of apoptosis. There is evidence that a loss of the physiological functions of PS1 may lead to apoptosis. For instance, it was demonstrated that inhibition of PS1 expression in cultured tumour cells or mice strains with spontaneous tumour development induces a higher rate of apoptosis (Roperch 1998). Since PS1 is proteolytically cleaved by caspase 3, the resulting C-terminal fragment was proposed to have a functional role in regulating apoptosis (Loetscher 1997; Kim 1997). Thus, the original idea in our lab was to clarify if the overexpression of full length PS1wt may reduce vulnerability of cells to apoptosis, and if the caspase 3 cleavage of PS1 is a functional step. Here it was expected that the mutation of the caspase 3 cleavage sequence in PS1 (PSmut) would avoid the antiapoptotic effect of PS1.

The results show that in stably-transfected PC12 cells, the overexpression of PS1wt has an antiapoptotic effect when oxidative stress was induced. Thus it is probably that one effect of FAD related PS1 mutations is the abolishment of this antiapoptotic effect leading to enhanced apoptotic rates. FAD mutated PS1 may cause early onset by triggering pro-apoptotic mechanisms since many reports have shown that different PS1 FAD mutations sensitize cell to apoptosis (Guo 1998; Tanii 2000; Zhou 2002; Chan 2002). In our lab this pro.apoptotic was observed in $CD4^+$-T-lymphocytes from mice bearing the FAD PS1 M146L mutation compared to PS1 wt mice $CD4^+$-T-lymphocytes (Schindowski 2003).This results suggest that PS1wt may have neuroprotective activity and that PS1 mutants may be defective in such neuroprotective function.

The caspase 3 cleaved C-terminal PS1 fragment (PSCas) show a similar effect as the full lengths PS1, therefore it would be attempting to speculate that the antiapoptotic effect of PS1 may be an inhibitory effect of the generated PSCas during apoptosis. However, the transfection of PSmut leads also to reduced apoptosis to similar levels as PS1 and PSCas, which now suggest that proteolysis of PS1 by caspase 3 may be not a determinant step for the antiapoptotic effect of PS1. Rather it seems that the C-terminal fragment of PS1 doesn't needs to be cleaved by caspase 3 for its antiapoptotic action. It would be interesting to construct a

N-terminal PS1 fragment. If this fragment show no antiapoptotic effect, this would confirm the hypothesis that only the C-terminal fragment of PS1 is essential for the antiapoptotic action, which is independent of its cleavage by caspase 3.

How to explain the antiapoptotic effect of PS1?

Due to several evidences of the implication of PS1 in intrinsic apoptotic pathway, the effects of PS1 overexpression on mitochondrial membrane potential and Bcl-xl expression were examinated. In addition, it was investigated if PS1 has an negative effect on JNK signalling pathway as it has been proposed recently by Kim et al (2001). Here the obtained results show that neither the mitochondrial membrane potential, nor the Bcl-xl expression are affected by the overexpression of the PS1 constructs, which leads to hypothesise that the antiapoptotic action of PS1 might not occur through intrinsic apoptosis, but rather due a different mechanism.

Possible is an interaction of PS1 with a proapoptotic factor, whereas the interaction of PS1 is mediated by the C-terminal Fragment. Here, e.g. the binding of PS1 could lead to an inactivation of this proapoptotic factor. An overexpression of PS1 may emphases the inactivation. Since the caspase cleavage sequence site is localized in the hydrophilic loop, the mutation of alanin to an aspartate may not affect the binding of PS1 to the proapoptotic factor. In contrast, FAD mutations are localized within or adjacent to the transmembrane domains. A mutation in these regions could lead to structural changes, affecting the binding interaction, liberating the proapoptotic factor. A candidate here is GSK3ß, which has been implicated in the pathological mechanism of AD. Recent work indicates that PS1 is able to interact with GSK3ß. Interestingly, GSK3ß activity were increased in embryonic neuronal cells and fibroblast cultures of PS1 knock-in M146V (KIM146V) and PS1 knockout mice (Pigino 2003). Thus, it will be interesting to measure if GSK3ß activity is reduced by PS1 wildtyp overexpression. The cell death mechanism mediated by GSK3ß was shown to be by the phosphorylation of kinesin light chains (KLC), causing the release of kinesin-I from membrane-bound organelles (MBOs). The result is an a reduction in kinesin-I driven motility (Morfini 2002b). These findings suggest that mutations in PS1 may compromise neuronal function by affecting GSK-3 activity and kinesin-I-based motility.

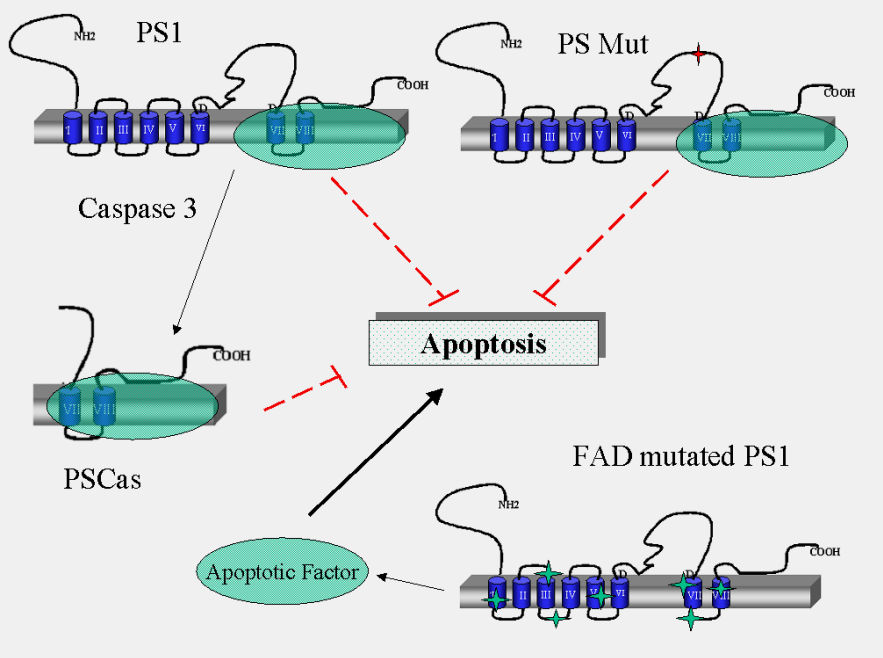

Fig. 4.4 A hypothetical mechanism of the antiapoptotic effect of the overexpression of PS1, PSCas and PSMut. It is possible that the overexpression of the PS1 constructs may bind or at least leads to an inactivation of an proapoptotic factor. The caspase 3 mutated cleavage sequence does not impair the binding of the apoptotic factor, since it is localized in the cytoplasmatic loop and might not lead to structural changes of in the C-terminal PS1 fragment. Since FAD mutations are localized within or adjacent to the transmembrane domains and have been shown to sensitise cells to apoptotic stimuli, it is probably that the FAD mutations result in structural changes of PS1, inhibiting the binding of the apoptotic factor and leading to its detachment, finally, leading to cell death.

5. Summary

Alzheimer's disease (AD) is the most common neurodegenerative disorder world wide, causing presenile dementia and death of millions of people. During AD damage and massive loss of brain cells occur. Alzheimer's disease is genetically heterogeneous and may therefore represent a common phenotype that results from various genetic and environmental influences and risk factors.

In approximately 10% of patients, changes of the genetic information were detected (gene mutations). In these cases, Alzheimer's disease is inherited as an autosomal dominant trait (familial Alzheimer's disease, FAD). In rare cases of familial Alzheimer's disease (about 1-3%), mutations have been detected in genes on chromosomes 14 and 1 (encoding for Presenilin 1 and 2, respectively), and on chromosome 21 encoding for the amyloid precursor protein (APP), which is responsible for the release of the cell-damaging protein amyloid-beta (ß-amyloid, Aß). Familial forms of early-onset Alzheimer's disease are rare; however, their importance extends far beyond their frequency, because they allow to identify some of the critical pathogenetic pathways of the disease. All familial Alzheimer mutations share a common feature: they lead to an enhanced production of the Aß, which is the major constituent of senile plaques in brains of AD patients.

New data indicates that Aß promotes neuronal degeneration. Therefore, one aim of these thesis was to elucidate the neurotoxic biochemical pathways induced by Aß, investigating the effect of the FAD Swedish APP double mutation (APPsw) on oxidative stress-induced cell death mechanisms. This mutation results in a three- to sixfold increased Aß production compared to wild-type APP (APPwt). As cell models, the neuronal PC12 (rat pheochromocytoma) and the HEK (human embryonic kidney 293) cell lines were used, which have been transfected with human wiltyp APP or human APP containing the Swedish double mutation. The used cell models offer two important advantages. First, compared to experiments using high concentrations of Aß at micromolar levels applied extracellularly to cells, PC12 APPsw cells secret low Aß levels similar to the situation in FAD brains. Thus, this cell model represents a very suitable approach to elucidate the AD-specific cell death pathways mimicking physiological conditions. Second, these two cell lines (PC12 and HEK APPwt and APPsw) with different production levels of Aß may additionally allow to study dose-dependent effects of Aß.

The here obtained results provide evidence for the enhanced cell vulnerability caused by the Swedish APP mutation and elucidate the cell death mechanism probably initiated by

intracellulary produced Aß. Here it seems likely that increased production of Aß at physiological levels primes APPsw PC12 cells to undergo cell death only after additional stress, while chronic high levels in HEK cells already lead to enhanced basal apoptotic levels. Crucial effects of the Swedish APP mutation include the impairments of cellular energy metabolism affecting mitochondrial membrane potential and ATP levels as well as the additional activation of caspase 2, caspase 8 and JNK in response to oxidative stress. Thereby ,the following model can be proposed: PC12 cells harboring the Swedish APP mutation have a reduced energy metabolism compared to APPwt or control cells. However, this effect does not leads to enhanced basal apoptotic levels of cultured cells. An exposure of PC12 cells to oxidative stress leads to mitochondrial dysfunction, e.g., decrease in mitochondrial membrane potential and depletion in ATP. The consequence is the activation of the intrinsic apoptotic pathway releasing cytochrome c and Smac resulting in the activation of caspase 9. This effect is amplified by the overexpression of APP, since both APPsw and APPwt PC12 cells show enhanced cytochrome c and Smac release as well as enhanced caspase 9 activity as vector transfected control. In APPsw PC12 cells a parallel pathway is additionally emphased. Due to reduced ATP levels or enhanced Aß production JNK is activated. Furthermore, the extrinsic apoptotic pathway is enhanced, since caspase 8 and caspase 2 activation was clearly enhanced by the Swedish APP mutation. Both pathways may then converge by activating the effector enzyme, caspase 3, and the execution of cell death. In addition, caspase independent effects also needs to be considered. One possibility could be the implication of AIF since AIF expression was found to be induced by the Swedish APP mutation. In APPsw HEK cells high chronic Aß levels leads to enhanced apoptotic levels, reduce mitochondrial membrane potential and ATP levels even under basal conditions.

Summarizing, a hypothetical sequence of events is proposed linking FAD, Aß production, JNK-activation, mitochondrial dysfunction with caspase pathway and neuronal loss for our cell model. The brain has a high metabolic rate and is exposured to gradually rising levels of oxidative stress during life. In Swedish FAD patients the levels of oxidative stress are increased in the temporal inferior cortex. This study using a cell model mimicking the in vivo situation in AD brains indicates that probably both, increased Aß production and the gradual rise of oxidative stress throughout life converge at a final common pathway of an increased vulnerability of neurons to apoptotic cell death from FAD patients.

Presenilin (PS) 1 is an aspartyl protease, involved in the γ-secretase mediated proteolysis of Amyloid-ß-protein (Aß), the major constituent of senile plaques in brains of Alzheimer's disease (AD) patients. Recent studies have suggested an additional role for presenilin proteins in apoptotic cell death observed in AD. Since PS 1 is proteolytic cleaved by caspase 3, it has been prosposed that the resulting C-terminal fragment of PS1 (PSCas) could play a role in signal transduction during apoptosis. Moreover, it was shown that mutant presenilins causing early-onset of familial Alzheimer's disease (FAD) may render cells vulnerable to apoptosis. The mechanism by which PS1 regulates apoptotic cell death is yet not understood. Therefore one aim of our present study was to clarify the involvement of PS1 in the proteolytic cascade of apoptosis and if the cleavage of PS1 by caspase 3 has an regulatory function. Here it is demonstrated that both, PS1 and PS1Cas lead to a reduced vulnerability of PC12 and Jurkat cells to different apoptotic stimuli. However a mutation at the caspase 3 recognition site (D345A/ PSmut), which inhibits cleavage of PS1 by caspase 3, show no differences in the effect of PS1 or PSCas towards apoptotic stimuli. This suggest that proteolysis of PS1 by caspase 3 is not a determinant, but only a secondary effect during apoptosis. Since several FAD mutation distributed through the whole PS1 gene lead to enhanced apoptosis, an abolishment of the antiapoptotic effect of PS1 might contribute to the massive neurodegeneration in early age of FAD patients. Here, the regulate properties of PS1 in apoptosis may not be through an caspase 3 dependent cleavage and generation of PSCas, but rather through interaction of PS1 with other proteins involved in apoptosis.

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6. Zusammenfassung

Massiver und fortschreitender neuronaler Zelltod gehört zu den zentralen Charakteristika der Alzheimer Demenz (AD), ebenso wie Senile Plaques und Neurofibrillenbündel. Die Aufklärung der Mechanismen, die zum neuronalen Zelltod führen, sind daher von entscheidender Bedeutung bei der Suche nach neuen therapeutischen Ansätzen. Verantwortlich für den neuronalen Zelltod ist das Amyloid-Beta Peptid (Aß), das ein proteolytisches Spaltprodukt des Amyloid Precursor Proteins (APP) ist. Die Spaltung von Aß erfolgt hierbei initial durch die ß-Sekretase und anschließend intermembranär durch den γ-Sekretase Komplex. Aß wird intrazellulär generiert und extrazellulär in aggregierten und unlöslichen Plaques angereichert. Die Mechanismen der Aß-Toxizität sind noch nicht genau bekannt; assoziiert werden oxidativer Stress, veränderte Kalziumhomöostase, mitochondriale Defekte und der Verlust an Überlebensfaktoren.

Weniger als 10% aller Fälle der AD werden dominant vererbt. Dies bedeutet, dass die Mutation eines einzigen Gens für die Entstehung der Krankheit ausreicht und dass statistisch gesehen die Hälfte der Nachkommen eines Betroffenen ebenfalls erkranken. Bisher sind drei Gene bekannt, die bei autosomal dominant vererbten Formen der AD (familiäre AD, FAD) mutiert sein können. Es handelt sich um die Gene Presenilin 1 und Presenilin 2 auf den Chromosomen 14 bzw. 1, sowie um das auf Chromosom 21 gelegene Gen für das Amyloid-Precursor-Molekül (APP). Die familiären Formen der AD sind selten, deren Bedeutung ragt jedoch weit über die Auftrittsfrequenz hinaus, weil sie es erlauben die kritische pathogenene Entwicklung der Krankheit in transgenen Modellen zu untersuchen. Alle familiären AD Mutationen haben eines gemeinsam; sie führen zu einer erhöhten Produktion von Aß.

Basierend auf Daten, die darauf hindeuten, dass Aß die neuronale Degeneration fördert, war es das Hauptziel der vorliegenden Arbeit, die neurotoxischen biochemischen Signaltransduktionswege von Aß zu untersuchen. Hierfür wurde der Effekt, der im Rahmen der FAD vorkommenden, schwedischen APP Mutation (APPsw), auf den durch oxidativen Stress induzierten Zelltodmechanismus untersucht. Diese Mutation resultiert in einer drei- bis sechsfach erhöhten Aß Produktion im Vergleich zum Wild-Typ APP (APPwt). Als Zellmodell wurden die neuronalen PC12- (rat pheochromocytoma) und die HEK- (human embryonic kidney 293) Zelllinien verwendet, die zuvor mit APPsw-, APPwt-, und als Kontrolle mit Leervektoren transfiziert wurden. Die verwendeten Zellmodelle bieten hierbei

zwei wichtige Vorteile. 1. Verglichen mit Experimenten, bei denen Aß extrazellulär im mikromolaren Bereich appliziert wird, sekretieren PC12 APPsw Zellen Aß im pikomolaren Bereich, entsprechend der physiologischen Produktion im Gehirn von AD Patienten. So stellt dieses Zellmodell eine sehr gute Annäherung an die tatsächlich physiologische Situation in Gehirnen von FAD Patienten dar. 2. Beide Zelllinien (PC12 und HEK, APPwt und APPsw) haben eine unterschiedliche Aß Produktion, was die Untersuchung von dosisabhängigen Aß-Effekten ermöglicht.

Die in dieser Arbeit gefundenen Ergebnisse zeigen, dass die Transfektion der schwedischen APP Mutation eine erhöhte Zellvulnerabilität bewirkt. Ebenso wurden die, wahrscheinlich von Aß induzierten, Zelltodmechanismen aufgeklärt. Interessanterweise konnte hierbei festgestellt werden, dass die unter physiologischen Bedingungen erhöhte Aß Produktion in PC12 Zellen erst dann zu einer erhöhte Apoptoserate führt, wenn eine sekundäre Stressinduktion stattfindet. Ein Einfluss der erhöhten APP Expression oder der erhöhten Aß Produktion auf die basale Apoptose (ohne sekundäre Stressinduktion) konnte in PC12 Zellen nicht festgestellt werden.
Die mit der schwedischen APP Mutation transfizierten HEK Zellen weisen eine 30fach erhöhte Aß Produktion im Vergleich zu den entsprechend transfizierten PC12 Zellen auf. Dies hat zur Folge, dass die chronisch erhöhte Aß Produktion in HEK Zellen auch schon unter basalen Bedingungen zu einer erhöhten Apoptoserate führt. Untersuchungen des mitochondrialen Membranpotentials und der zellulären ATP Spiegel zeigten, dass die ausschlaggebenden Effekte der schwedischen APP Mutation die Beeinträchtigung des zellulären Energiemetabolismus beinhalten. Sowohl basale ATP Spiegel als auch ein reduziertes mitochondriales Membranpotential wurden in APPsw- transfizierten HEK Zellen im Vergleich zu den APPwt Zellen gemessen. Auch APPsw- transfizierte PC12 Zellen haben einen reduzierten Energiemetabolismus wie die Untersuchung der ATP-Spiegel und der metabolischen Aktivität zeigten. Dies wird aber unter basalen Bedingungen jedoch anscheinend kompensiert.

Ein zentraler Aspekt der vorliegenden Arbeit war es, die Frage zu beantworten, warum die Überexpression von APP zu einer erhöhten Vulnerabilität gegenüber oxidativen Stress bei PC12 Zellen führt. Auch sollte geklärt werden, wie die Überexpression der schwedischen APP Mutation diesen Effekt noch zusätzlich verstärkt. Hierfür wurden verschiedene Signaltransduktionswege des apoptotischen Zelltods untersucht. Wie es sich herausstellte,

scheint die Beeinträchtigung der mitochondrialen Funktion eine wesentliche Auswirkung der APP-Überexpression zu sein. Eine Induktion von oxidativem Stress führte in PC12 Zellen zu einer Aktivierung der intrinsischen Apoptosekaskade, mit einer Freisetzung von Cytochrom C und Smac, sowie einer resultierenden Aktiverung der Initiatorcaspase 9. Die intrinsische Apoptose wird hierbei durch die Überexpression von APP verstärkt. Sowohl APPsw als auch APPwt PC12 Zellen wiesen eine erhöhte Cytochrom C- und Smac- Freisetzung in das Cytosol auf, im Vergleich zu Vector-transfizierten PC12 Zellen. Ebenso wurde eine erhöhte Caspase 9 Aktivität bei APPsw- und APP-transfizierten Zellen im Vergleich zu den Vektor-transfizierten Zellen gemessen.

Weitere Untersuchungen zeigten, dass die schwedische APP Mutation darüber hinaus noch eine zusätzliche Aktivierung von Caspase 2 und Caspase 8 bewirkt wenn PC12 Zellen oxidativem Stress ausgesetzt sind. Eine deutlich erhöhte Caspase 2- und Caspase 8-Aktivität konnte in APPsw- transfizierten Zellen im Vergleich zu APPwt PC12 Zellen gemessen werden. Da dies zwei Initiatorcaspasen sind, die normalerweise extrazelluläre Zelltodsignale vermitteln, deutet das auf eine Aktivierung der extrinsischen Apoptose.
Ebenso konnte gezeigt werden, dass sowohl in HEK Zellen als auch in PC12 Zellen die Induktion von oxidativem Stress zu einer Aktivierung der c-Jun terminalen Kinase (JNK) führt. Interessanterweise konnte gefunden werden, dass die Aktivierung der JNK durch die schwedische APP Mutation verstärkt wird, was auf eine wichtige Rolle der JNK im Rahmen der Aß Toxizität hindeutet. In PC12 Zellen konnte zusätzlich beobachtet werden, dass die Inhibierung von JNK mit SP600125, einem spezifischen JNK Inhibitor, Mitochondrien vor dem, durch oxidativen Stress induzierten, Zusammenbrechen des Membranpotential schützt. Auch konnte aktiviertes JNK in der mitochondrialer Fraktion nachgewiesen werden, was ebenso darauf hindeutet, dass JNK zusätzlich zu der Aktivierung des Transkriptionsfaktors c-Jun auch Signale zu Mitochondrien vermittelt.

Ausgehend von diesen Ergebnissen kann die erhöhte Vulnerabilität der APPsw- transfizierten Zellen bei der Induktion von oxidativen Stress folgendermaßen nachvollzogen werden:
APPsw- transfizierte PC12 Zellen haben einen reduzierten Energiemetabolismus im Vergleich zu APPwt- und Vektor- transfizierten PC12 Zellen. Der beeinträchtigte Zellmetabolismus wird anscheinend jedoch kompensiert, da dieser Effekt alleine nicht zu einer erhöhten basalen Apoptoserate führt. Eine zusätzliche Induktion von oxidativem Stress führt dann zu einen weiteren Abfall des mitochondrialen Membranpotentials und der ATP Spiegel. Die Folge ist

eine Aktivierung der intrinsichen Apoptosekaskade, mit einer Freisetzung von Cytochrom C und Smac und einer Aktivierung der Caspase 9. Diese Effekte werden hierbei durch die Überexpression von APP verstärkt. Die schwedische APP Mutation führt gleichzeitig aber auch zur Verstärkung parallel verlaufender Signale. Aufgrund der reduzierten ATP-Spiegel oder der erhöhten Aß Produktion findet eine zusätzliche Aktivierung der JNK statt. Ebenso findet zusätzlich eine verstärkte Aktivierung der extrinsischen Apoptosekaskade statt, da eine deutlich erhöhte Caspase 8- und Caspase 2- Aktivierung bei der Induktion von oxidativem Stress in APPsw PC12 Zellen auftritt. Beide Signalwege führen zur Aktivierung der Caspase 3, die als Effektor Caspase den Zelltod vollstreckt. Zusätzlich müssen aber auch noch Caspase unabhängige Effekte berücksichtigt werden. Eine Möglichkeit besteht in der Aktivierung von AIF, da eine verstärkte AIF Expression induziert durch die schwedische APP Mutation nachgewiesen wurde. In APPsw transfizierten HEK Zellen führen chronisch erhöhte Aß Spiegel selbst unter basalen Bedingungen zu einer erhöhten Apoptoserate, einem reduzierten mitochondrialen Membranpotential und reduzierten ATP Spiegeln.

Zusammenfassend wird aus den hier erzielten Ergebnissen eine hypothetische Sequenz vorgeschlagen, wie es zum frühen Einsetzen der FAD kommen könnte: das Gehirn hat eine hohe metabolische Aktivität und ist im Laufe des Lebens einer zunehmenden Menge an oxidativem Stress ausgesetzt. Bei Patienten, die Träger der schwedischen APP Mutation sind, wurden in der Hirnrinde erhöhte Level an oxidativen Stress festgestellt. Die im Rahmen dieser Arbeit durchgeführten Versuche, zeigen, dass sowohl eine erhöhte Aß Produktion als auch die zunehmende Menge an oxidativem Stress zu einer erhöhten Vulnerabilität von Neuronen bei FAD Patienten führt. Entscheidend sind hierbei die Aktivierungen von Caspase 2, Caspase 8 und JNK Dies hat ein verstärktes Absterben von Neuronen durch den apoptotischen Zelltod und ein frühes Eintreten von Morbus Alzheimer zur Folge.

Im Rahmen der familiären Alzheimer Erkrankung wurden bisher über 120 Mutationen im Presenilin 1 Gen gefunden. Ebenso wie bei Mutationen innerhalb des APP Gens führen auch die Presenilin Mutationen zu einer erhöhten Aß Bildung. Wie vor kurzem nachgewiesen werden konnte, ist Presenilin 1 ein transmembranäres Protein mit 6-8 Transmembrandomänen und ein Bestandteil des γ-Sekretase Komplexes. Ebenso wie die ß-Sekretase ist die γ-Sekretase an der Spaltung des Aß-Peptids aus dem Vorläuferprotein APP beteiligt. Die Spaltung von Aß erfolgt hierbei initial durch die ß-Sekretase und anschließend intermembranär durch den γ-Sekretase Komplex. Presenilin 1 wirkt hierbei im katalytischen

Zentrum des Komplexes als Protease. Weitere Bestandteile des Komplexes sind Nicastrin, Pen-2 und APH-1. Diese Komponenten sind ebenfalls für die γ-Sekretase Aktivität essentiell, wobei sie als Co-Faktoren eine wichtige Rolle bei der Bildung des Multiproteinkomplexes erfüllen. Das Ausschalten einer dieser Faktoren führt zum Aktivitätsverlust.

Wie Presenilin 1 FAD Mutationen zu einer gesteigerten Aß Produktion und einem frühen Beginn der Alzheimer Erkrankung führen ist unbekannt. Interessenterweise sind alle bekannten FAD Mutation in den transmembranären Regionen lokalisiert. Zwischen den Transmembrandomänen 6 und 7 existiert ein großer hydrophiler Loop, der endoproteolytisch gespalten wird. Ebenfalls lokalisiert ist hier eine Caspase 3 Erkennungssequenz. Die Spaltung von Presenilin 1 durch Caspase 3 resultiert in der Bildung eines C-terminalen Fragments. Eine Rolle für Presenilin im Rahmen des apoptotischen Zelltods ist daher annehmbar. So wurde postuliert, dass das resultierende C-terminale Fragment von PS1 (PSCas) eine Rolle während der apoptotischen Signaltransduktion haben könnte. Ebenfalls wurde gezeigt, dass FAD mutiertes PS1 zu einer erhöhten Vulnerabilität führen kann. Im Rahmen der vorliegenden Arbeit wurde daher untersucht, ob Presenilin 1 einen Einfluss auf die Zellvulnerabilität und die apoptotischen Zelltodkaskasde ausübt. Ebenfalls wurde eine mögliche Rolle des PSCas Fragments untersucht. Hierzu wurde ausgehend von einem Presenilin 1 Plasmid ein PSCas Konstrukt hergestellt. Ein weiteres Konstrukt wurde erzeugt, indem Presenilin 1 an der Caspase 3 Erkennungssequenz mutiert wurde, was die Spaltung von PS1 durch Caspase 3 verhindert (PSMut).

Die Transfektionsversuche in stabil transfizierten PC12 und transient transfizierten Jurkat Zellen ergaben, dass die Überexpression von Presenilin 1 eine antiapoptotische Wirkung bewirkt. Sowohl bei dem durch oxidativen Stress induzierten Zelltod, als auch bei der Induktion von Apoptose mit Fas-Ligand zeigten Zellen mit der Presenilin 1 Überexpression eine geringere Vulnerabilität im Vergleich zu Vektor- transfizierten Kontrollen. In Anbetracht der aktuellen Forschungsergebnisse, die auf eine Rolle von PSCas in der Regulation der Apoptose hindeuteten, war die Annahme zu Beginn dieser Arbeit, dass die antiapoptotische Wirkung des Presenilin 1 auf das PSCas Fragment zurückzuführen ist. Wie die Ergebnisse der PSCas Überexpression zeigen, scheint dies auch der Fall zu sein. Ähnlich der Überexpression von Presenilin 1 führt die alleinige Überexpression von PSCas zu einer reduzierten Zellvulnerabilität im Vergleich zu Vektor- transfizierten Kontrollen. In der Annahme, dass die Spaltung von Presenilin 1 hier eine regulatorische Funktion besitzten würde, erwartete man bei der Überexpression von PSMut eine Aufhebung der antiapoptotischen Wirkung. Wie sich

jedoch herausstellte, führt auch die Überexpression von Presenilin 1 mit mutierter Caspase 3 Erkennungssequenz zu einer verminderten Zellvulnerabilität. Dies deutet darauf hin, dass die antiapoptotische Wirkung von Presenilin 1 auf den C-terminalen Bereich des Proteins zurückzuführen ist, hierfür aber keine Spaltung durch Caspase 3 notwendig ist. Die Spaltung von Presenilin 1 durch Caspase 3 besitzt wahrscheinlich daher keine Funktion bei der Regulation der Apoptose. Eher ist hier anzunehmen, dass Presenilin 1 eines der vielen Substrate von Caspase 3 im späten Stadium der Apoptosekaskade darstellt. Basierend auf Ergebnissen, die zeigen, dass FAD mutiertes Presenilin zu einer erhöhten Zellvulnerabilität führt, könnte in der Aufhebung der antiapoptotischen Presenilin 1 Wirkung die Ursache des frühen Erkrankungsbeginns liegen.

7. References:

Alberici A, Moratto D, Benussi L, Gasparini L, Ghidoni R, Gatta LB, Finazzi D, Frisoni GB, Trabucchi M, Growdon JH, Nitsch RM, Binetti G. Presenilin 1 protein directly interacts with Bcl-2. J Biol Chem. 1999 Oct 22;274(43):30764-9.

Aliev G, Seyidova D, Lamb BT, Obrenovich ME, Siedlak SL, Vinters HV, Friedland RP, LaManna JC, Smith MA, Perry G. Mitochondria and vascular lesions as a central target for the development of Alzheimer's disease and Alzheimer disease-like pathology in transgenic mice. Neurol Res. 2003 Sep; 25(6): 665-74.

Alzheimer A. Über eine eigenartige Erkrankung der Hirnrinde. Allg Z Psychiatr Psych-Gerichtl Med 1907 64, 146-8; 1907.

Amson R, Lassalle JM, Halley H, Prieur S, Lethrosne F, Roperch JP, Israeli D, Gendron MC, Duyckaerts C, Checler F, Dausset J, Cohen D, Oren M, Telerman A (2000) Behavioral alterations associated with apoptosis and down-regulation of presenilin 1 in the brains of p53-deficient mice. Proc Natl Acad Sci USA 97:5346–5350.

Anandatheerthavarada HK, Biswas G, Robin MA, Avadhani NG. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid precursor protein impairs mitochondrial function in neuronal cells. J Cell Biol. 2003 Apr 14;161(1):41-54.

Aoki H, Kang PM, Hampe J, Yoshimura K, Noma T, Matsuzaki M, Izumo S. Direct activation of mitochondrial apoptosis machinery by c-Jun N-terminal kinase in adult cardiac myocytes. J Biol Chem. 2002 Mar 22; 277(12): 10244-50.

Arch, R. H., Gedrich, R. W., and Thompson, C. B. (1998). Tumor necrosis factor receptor-associated factors (TRAFs)—A family of adapter proteins that regulates life and death. *Genes Dev.* **12,**2821–2830.

Atwood CS, Martins RN, Smith MA, Perry G (2002) Senile plaque composition and posttranslational modification of amyloid-beta peptide and associated proteins. Peptides 23: 1343-1350

Barnhart BC, Lee JC, Alappat EC, Peter ME.The death effector domain protein family. Oncogene. 2003 Nov 24; 22(53): 8634-44.

Bayer TA, Schafer S, Simons A, Kemmling A, Kamer T, Tepests R, Eckert A, Schussel K, Eikenberg O, Sturchler-Pierrat C, Abramowski D, Staufenbiel M, Multhaup G. Dietary Cu stabilizes brain superoxide dismutase 1 activity and reduces amyloid Abeta production in APP23 transgenic mice. Proc Natl Acad Sci U S A. 2003 Nov 25; 100(24): 14187-92

Beffert U, Poirier J Apolipoprotein E, plaques, tangles and cholinergic dysfunction in Alzheimer's disease. Ann N Y Acad Sci 1996, 777: 166-174.

Belzacq AS, El Hamel C, Vieira HL, Cohen I, Haouzi D, Metivier D, Marchetti P, Brenner C, Kroemer G. Adenine nucleotide translocator mediates the mitochondrial membrane permeabilization induced by lonidamine, arsenite and CD437. Oncogene. 2001 Nov 15;20(52):7579-87.

Belzacq AS, Vieira HL, Verrier F, Vandecasteele G, Cohen I, Prevost MC, Larquet E, Pariselli F, Petit PX, Kahn A, Rizzuto R, Brenner C, Kroemer G. Bcl-2 and Bax modulate adenine nucleotide translocase activity. Cancer Res. 2003 Jan 15;63(2):541-6.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):13681-6.

Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys. 1993 Jun;303(2):474-82.

Bertram L, Blacker D, Mullin K, Keeney D, Jones J, Basu S, Yhu S, McInnis MG, Go RC, Vekrellis K, Selkoe DJ, Saunders AJ, Tanzi RE, Evidence for genetic linkage of Alzheimer's disease to chromosome 10q, Science 290 (2000) 2302–2303.

Bidere, N. & Senik, A. (2001) Caspase-independent apoptotic pathways in T lymphocytes: a minireview. Apoptosis, 6, 371±375.

Binder LI, Frankfurter A, Rebhun LI. The distribution of tau in the mammalian central nervous system. J Cell Biol. 1985 Oct;101(4):1371-8.

Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B, Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE, Alpha-2 macroglobulin genetically associated with Alzheimer disease, Nat. Genet. 19 (1998) 357–360.

Blass JP, Gibson GE, Hoyer S. The role of the metabolic lesion in Alzheimer's disease. J Alzheimers Dis. 2002 Jun;4(3):225-32.

Bogdanovic N (2000) Alzheimer's disease: plaques and tangles J.Cell.Mol.Med. Vol 4, No 4, 2000. 304-305

Bogdanovic N, Zilmer M, Zilmer K, Rehema A, Karelson E. The Swedish APP670/671 Alzheimer's disease mutation: the first evidence for strikingly increased oxidative injury in the temporal inferior cortex. Dement Geriatr Cogn Disord. 2001 Nov-Dec; 12(6): 364-70.

Bonelli RM, Wenning GK, Kapfhammer HP. Huntington's disease: present treatments and future therapeutic modalities. Int Clin Psychopharmacol. 2004 Mar;19(2):51-62.

Braak H., Braak E. (1994) Pathology of Alzheimer's disease. In: Calne D.B. ed. Neurodegenerative diseases. Philadelphia: Saunders: 585-613

Bratton SB, MacFarlane M, Cain K, Cohen GM. Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis Exp Cell Res. 2000 Apr 10;256(1):27-33.

Bue´e-Scherrer V, Goedert M. Phosphorylation of microtubule-associated protein Tau by stress-activated protein kinases in intact cells.FEBS Lett 2002;515:151–4.

Burns MP, Noble WJ, Olm V, Gaynor K, Casey E, LaFrancois J, Wang L, Duff K (2003) Colocalization of cholesterol, apolipoprotein E and fibrillar Abeta in amyloid plaques. Brain ResMol Brain Res 110: 119-125

Butterfield DA, Hensley K, Harris M, Mattson M, Carney J. Beta amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. Biochem Biophys Res Commun 1994;200:710–5.

Buxbaum JD, Liu KN, Luo Y, Slack JL, Stocking KL, Peschon JJ, Johnson RS, Castner BJ, Cerretti DP, Black RA. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated

alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J Biol Chem. 1998 Oct 23; 273(43): 27765-7.

Brown GC, Borutaite V. Nitric oxide inhibition of mitochondrial respiration and its role in cell death. Free Radic Biol Med. 2002 Dec 1;33(11):1440-50

Cai,X.D.; Golde,T.E.; Younkin,S.G. Release of excess amyloid beta protein from a mutant amyloid beta protein precursor (1993) *Science* **259,** 514-516

Cain K. Chemical-induced apoptosis: formation of the Apaf-1 apoptosome. Drug Metab Rev. 2003 Nov; 35(4): 337-63.

Campion D, Dumanchin C, Hannequin D. Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity and mutation spectrum. Am Hum Genet 1999;65:664-70.

Cande C, Cecconi F, Dessen P, Kroemer G.Apoptosis-inducing factor (AIF): key to the conserved caspase-independent pathways of cell death? J Cell Sci. 2002 Dec 15; 115(Pt 24): 4727-34.

Cantarella G, Uberti D, Carsana T, Lombardo G, Bernardini R, Memo M.Neutralization of TRAIL death pathway protects human neuronal cell line from beta-amyloid toxicity. Cell Death Differ. 2003 Jan;10(1):134-41.

Cao X., Sudhof T.C, A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60, Science 293 (2001) 115–120.

Capell A, Grunberg J, Pesold B, Diehlmann A, Citron M, Nixon R, Beyreuther K, Selkoe DJ, Haass C. The proteolytic fragments of the Alzheimer's disease-associated presenilin-1 form heterodimers and occur as a 100-150-kDa molecular mass complex. (1998) *J.Biol.Chem.* **273,** 3205-3211

Cardoso SM, Proenca MT, Santos S, Santana I, Oliveira CR.Cytochrome c oxidase is decreased in Alzheimer's disease platelets. Neurobiol Aging. 2004 Jan;25(1):105-10.

Cedazo-Minguez A, Popescu BO, Blanco-Millan JM, Akterin S, Pei JJ, Winblad B, Cowburn RF. Apolipoprotein E and beta-amyloid (1-42) regulation of glycogen synthase kinase-3beta. J Neurochem. 2003 Dec; 87(5): 1152-64.

Chomczynski P, Sacchi N.Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987 Apr;162(1):156-9.

Chishti,M.A.; Yang,D.S.; Janus,C.; Phinney,A.L.; Horne,P.; Pearson,J.; Strome,R.; Zuker,N.; Loukides,J.; French,J.; Turner,S.; Lozza,G.; Grilli,M.; Kunicki,S.; Morissette,C.; Paquette,J.; Gervais,F.; Bergeron,C.; Fraser,P.E.; Carlson,G.A.; George-Hyslop,P.S.; Westaway,D. Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695(2001) *J.Biol.Chem.* **276,** 21562-21570

Chui DH, Tanahashi H, Ozawa K, Ikeda S, Checler F, Ueda O, Suzuki H, Araki W, Inoue H, Shirotani K, Takahashi K, Gallyas F, Tabira T: Transgenic mice with Alzheimer presenilin 1 mutations show accelerated neurodegeneration without amyloid plaque formation. Nat Med 1999;5:560–564.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J..(1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer’s disease increases beta-protein production. Nature 360: 672-674

Cleveland DW, Hwo SY, Kirschner MW: Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. J Mol Biol 1977, 116:207-225.

Cooper AJL. Glutathione in the brain: disorders of glutathione metabolism. In: Rosenberg RN, Prusiner SB, DiMauro S, Barchi RL, Klunk LM, eds. The molecular and genetic basis of neurological disease. Boston: Butterworth-Heinemann, 1997:1242–5.

Corder E.H., Saunders A.M., Strittmatter W.J., Schmechel D.E.,.Gaskell P.C, Small G.W., Gene dose of apolipoprotein E type ε4 allele and the risk of Alzheimer's disease in late onset families, Science 261 (1993) 921–923.

Cory S and Adams JM. The Bcl2 family: regulators of the cellular life-or death switch. Nat Rev Cancer 2: 647_656, 2002.

Chen M, Ona VO, Li M, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med 2000;6:797-801.

Cheng Y, Deshmukh M, D'Costa A, Demaro JA, Gidday JM, Shah A, Sun Y, Jacquin MF, Johnson EM and Holtzman DM (1998) Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxicischemic brain injury [see comments]. J. Clin. Invest. 101: 1992 ± 1999

Christen Y. Oxidative stress and Alzheimer disease. Am J Clin Nutr 2000;71:621S–9S.

Chishti, M. A., Yang, D. S., Janus, C., Phinney, A. L., Horne, P., Pearson, J., Strome, R., Zuker, N., Loukides, J., French, J., Turner, S., Lozza, G., Grilli, M., Kunicki, S., Morissette, C., Paquette, J., Gervais, F., Bergeron, C., Fraser, P. E., Carlson, G. A., George-Hyslop, P. S., and Westaway, D. (2001) J.Biol.Chem. 276, 21562-21570

Cruts, M., van Duijn, C. M., Backhovens, H., Van den, B. M., Wehnert, A., Serneels, S., et al. (1998) Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease. Hum. Mol. Genet. 7, 43–51.

Cruz JC, Tseng HC, Goldman JA, Shih H, Tsai LH.Aberrant Cdk5 activation by p25 triggers pathological events leading to neurodegeneration and neurofibrillary tangles. Neuron. 2003 Oct 30;40(3):471-83

Daemen MA, van `t Veer C, Denecker G, Heemskerk VH,Wolfs TG, Clauss M, Vandenabeele P and Buurman WA (1999) Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. J. Clin. Invest. 104: 541 ± 549

M. Dahiyat, A. Cumming, C. Harrington, C. Wischik, J. Xuereb, F. Corrigan, G. Breen, D. Shaw, D. St Clair, Association between Alzheimer's disease and the NOS3 gene, Ann. Neurol. 46 (1999) 664–667.

Da Rocha AB, Mans DR, Regner A, Schwartsmann G. Targeting protein kinase C: new therapeutic opportunities against high-grade malignant gliomas? Oncologist 2000.;7(1):17-33

Daugas, E., Nochy, D., Ravagnan, L., Loefler, M., Susin, S.A., Zamzami, N. Kroemer, G. (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett., 476, 118±123.

De la Monte S.M., Bloch K.D., Aberrant expression of the constitutive endothelial nitric oxide synthase gene in Alzheimer disease, Mol. Chem. Neuropathol. 30 (1997) 139–159.

De la Monte SM, Chiche J, von dem Bussche A, Sanyal S, Lahousse SA, Janssens SP, Bloch KD. Nitric oxide synthase-3 overexpression causes apoptosis and impairs neuronal mitochondrial function: relevance to Alzheimer's-type neurodegeneration. Lab Invest. 2003 Feb;83(2):287-98.

Deng Y, Ren X, Yang L, Lin Y, Wu X. A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell. 2003 Oct 3;115(1):61-70.

De Strooper B. Aph-1, Pen-2, and Nicastrin with Presenilin Generate an Active γ-Secretase Complex Neuron Vol. 38, 9–12, April 10, 2003,

Dickson DW, Crystal HA, Bevona C, Honer W, Vincent I, Davies P. 1995. Correlations of synaptic and pathological markerswith cognition of the elderly. Neurobiol.Aging 16:285–98

Dietrich N, Thastrup J, Holmberg C, Gyrd-Hansen M, Fehrenbacher N, Lademann U, Lerdrup M, Herdegen T, Jaattela M, Kallunki T. JNK2 mediates TNF-induced cell death in mouse embryonic fibroblasts via regulation of both caspase and cathepsin protease pathways. Cell Death Differ. 2003 Dec 12

Doan A., Thinakaran G., Borchelt D.R., Slunt H.H., Ratovitsky T., Podlisny M., Selkoe D.J., Seeger M., Gandy S.E., Price D.L., Sisodia S.S., Protein topology of presenilin 1, Neuron 17:1023-1030, 1996

Doyle CA, Slater P. Localization of neuronal and endothelial nitric oxide synthase isoforms in human hippocampus, Neuroscience 76 (1997) 387–395.

Droet B, Pincon-Raymond M, Chambaz J, Pillot T (1999) Laminin 1 attenuates beta-amyloid peptide (1-40) neurotoxicity of cultured fetal rat cortical neurons. J Neurochem 73: 742-749

Earnshaw WC, Martins LM, Kaufmann SH.Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem. 1999;68:383-424.

Eckert A, Steiner B, Marques C, Leutz S, Romig H, Haass C, Muller WE. Elevated vulnerability to oxidative stress-induced cell death and activation of caspase-3 by the Swedish amyloid precursor protein mutation. J Neurosci Res. 2001 Apr 15;64(2):183-92.

Eckert A, Schindowski K, Leutner S, Luckhaus C, Touchet N, Czech C, Muller WE. Alzheimer's disease-like alterations in peripheral cells from presenilin-1 transgenic mice. Neurobiol Dis. 2001b Apr;8(2):331-42.

Eckert A, Keil U, Marques CA, Bonert A, Frey C, Schussel K, Muller WE. Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. Biochem Pharmacol. 2003 Oct 15;66(8):1627-34.

Eckert GP, Kirsch C, Mueller WE Differential effects of lovastatin treatment on brain cholesterol levels in normal and apoE-deficient mice. Neuroreport. 2001 Apr 17; 12(5): 883-7.

Eckert GP, Kirsch C, Müller WE (2003) Brain-membrane cholesterol in Alzheimer´s disease. J Nutr Health Aging 7: 18-23

Edbauer, D., Willem, M., Lammich, S., Steiner, H., Haass, C. (2002). Insulin-degrading enzyme rapidly removes the beta-amyloid precursor protein intracellular domain (AICD). The Journal of Biological Chemistry, 277, 13389–13393.

Facchinetti F, Furegato S, Terrazzino S, Leon A. H(2)O(2) induces upregulation of Fas and Fas ligand expression in NGF-differentiated PC12 cells: modulation by cAMP. J Neurosci Res. 2002 Jul 15;69(2):178-88.

Forloni G, Chiesa R, Smiroldo S, Verga L, Salmona M, Tagliavini F, Angeretti N. Apoptosis mediated neurotoxicity induced by chronic application of beta amyloid fragment 25–35. Neuroreport 1993;4:523–6.

Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S, Zecchi Orlandini S (2000) Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. J Cell Physiol 182:41–49

Frame S, Cohen P (2001) GSK3 takes centre stage more than 20 years after its discovery. Biochem J 359:1–16.

Frame S, Cohen P, Biondi RM: A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol Cell 2001, 7:1321-1327.

Francis R, McGrath G, Zhang J, Ruddy DA, Sym M, et al. 2002. Aph-1 and Pen-2 are required for Notch pathway signaling, gammasecretase cleavage of betaAPP, and presenilin protein accumulation. Dev. Cell 3:85–97

Gau, J. T., Steinhilb, M. L., Kao, T. C., D'Amato, C. J., Gaut, J. R., Frey, K. A., and Turner, R. S.(2002) Am.J.Pathol. 160, 731-738

Garcia ML, Cleveland DW. Going new places using an old MAP: tau, microtubules and human neurodegenerative disease. Curr Opin Cell Biol. 2001 Feb;13(1):41-8.

Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg LH, Ruffolo SC, Thornberry NA, Xanthoudakis S, Zamboni RJ, Roy S, Nicholson DW.Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic A beta peptide formation. Cell. 1999 Apr 30;97(3):395-406.

Ghahremani MH, Keramaris E, Shree T, Xia Z, Davis RJ, Flavell R, Slack RS, Park DS. Interaction of the c-Jun/JNK pathway and cyclin-dependent kinases in death of embryonic cortical neurons evoked by DNA damage. J Biol Chem. 2002 Sep 20;277(38):35586-96. Epub 2002 Jun 28.

Gibson GE, Sheu KF, Blass JP.Abnormalities of mitochondrial enzymes in Alzheimer disease. J Neural Transm. 1998;105(8-9):855-70.

Gibson GE. Interactions of oxidative stress with cellular calcium dynamics and glucose metabolism in Alzheimer's disease (1,2). Free Radic Biol Med 2002;12(32):1061–70.

Goate A, Chartier-Harlin Mc, Mullan M, Brown J, Crawford F, Fidani L, Guiffra L, Haynes A, Irving N, James L, Mant R, Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, And Hardy J. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349: 704–706, 1991.

Gottlieb,R.A.; Granville,D.J. Analyzing mitochondrial changes during apoptosis. Methods 2002; 26 (4), 341.347

Goutte C, Tsunozaki M, Hale VA, Priess JR. 2002. APH-1 is a multipass membrane protein essential for the Notch signaling pathway in Caenorhabditis elegans embryos. Proc. Natl. Acad. Sci. USA 99:775–79

Gray N, Detivaud L, Doerig C, Meijer L. ATP-site directed inhibitors of cyclin-dependent kinases. Curr Med Chem.1999 Sep;6(9):859-75.

Greene LA, Tischler AS Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve grow factor. Proc Natl Acad Sci USA 1976 73: 2424-2428.

Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998 Aug 28; 281(5381): 1309-12.

Grewal SS, York RD, Stork PJ: Extracellularsignal-regulated kinase signalling in neurons. Curr Opin Neurobiol 1999;9:544–553.

Grunberg J, Walter J, Loetscher H, Deuschle U, Jacobsen H, Haass C. Alzheimer's disease associated presenilin-1 holoprotein and its 18-20 kDa C-terminal fragment are death substrates for proteases of the caspase family. Biochemistry. 1998 Feb 24;37(8):2263-70.

Guo Q, Sopher BL, Furukawa K, Pham DG, Robinson N, Martin GM, Mattson MP. 1997. Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid betapeptide: involvement of calcium and oxyradicals. J Neurosci 17:4212–4222.

Guo Q, Fu W, Sopher BL, Miller MW, Ware CB, Martin GM, Mattson MP. Increased vulnerability of hippocampal neurons to excitotoxic necrosis in presenilin-1 mutant knock-in mice. Nat Med. 1999 Jan;5(1):101-6.

Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T and Alnemri ES (2002) Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. J. Biol. Chem. 277: 13430–13437

Haass C, Koo EH, Mellon A, Hung AY, Selkoe DJ.Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. Nature. 1992 Jun 11;357(6378):500-3.

Haass C, Hung AY, Selkoe DJ, and Teplow DB. Mutations associated with a locus for familial Alzheimer's disease result in alternative processing of amyloid b-protein precursor. J Biol Chem 269: 17741–17748, 1994.

Haass,C.; Lemere,C.A.; Capell,A.; Citron,M.; Seubert,P.; Schenk,D.; Lannfelt,L.; Selkoe,D.J. The Swedish mutation causes early-onset Alzheimer's disease by beta- secretase cleavage within the secretory pathway (1995) *Nat.Med.* **1,** 1291-1296

Haass C, Steiner S 2002 Alzheimer disease γ-secretase: a complex story of GxGD-type presenilin proteases TRENDS in Cell Biology Vol.12 No.12

Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet. 1994 Sep 10;344(8924):721-4

Hardy JA, Higgins GA: Alzheimer's disease:the amyloid cascade hypothesis. Science 1992; 256:184–185.

Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 2002 Jul 19;297(5580):353-6.

Harman D. Free radical theory of aging. Mutat Res 1992;275:257–66.

Harris CA, Deshmukh M, Tsui-Pierchala B, Maroney AC, Johnson EM Jr. Inhibition of the c-Jun N-terminal kinase signaling pathway by the mixed lineage kinase inhibitor CEP-1347 (KT7515) preserves metabolism and growth of trophic factor-deprived neurons. J Neurosci. 2002 Jan 1;22(1):103-13.

Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, Roberts GW, Masters CL, Dotti CG, Unsicker K, BeyreutherK. Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides. Nat Med. 1997 Sep;3(9):1016-20.

Hartmann T. Intracellular biology of Alzheimer's disease amyloid beta peptide. Eur Arch Psychiatry Clin Neurosci 1999;249:291–8.

Hashimoto Y, Yuko I,1 Arakawa E, Kita Y, Terashita K, Niikura T, Nishimoto I. Neurotoxic mechanisms triggered by Alzheimer's disease-linked mutant M146L presenilin 1: involvement of NO synthase via a novel pertussis toxin target. Journal of Neurochemistry, 2002, 80, 426-437

Hashimoto Y, Niikura T, Chiba T, Tsukamoto E, Kadowaki H, Nishitoh H, Yamagishi Y, Ishizaka M, Yamada M, Nawa M, Terashita K, Aiso S, Ichijo H, Nishimoto I.The cytoplasmic domain of Alzheimer's amyloid-beta protein precursor causes sustained apoptosis signal-regulating kinase 1/c-Jun NH2-terminal kinase-mediated neurotoxic signal via dimerization. J Pharmacol Exp Ther. 2003 Sep;306(3):889-902

Hensley K, Carney JM, Mattson MP, Aksenova M, Harris M, Wu JF, Floyd RA, Butterfield DA. 1994. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer's disease. Proc Natl Acad Sci USA 91:3270 –3274.

Herdegen T, Skene P, Bahr M. The c-Jun transcription factor, bipotential mediator of neuronal death, survival and regeneration. Trends Neurosci. 1997 May; 20(5): 227-31.

Hetts SW (1998) To die or not to die: an overview of apoptosis and its role in disease JAMA 279: 300 ± 307

Holcomb LA, Gordon MN, Jantzen P, Hsiao K, Duff K, Morgan D: Behavioral changes in transgenic mice expressing both amyloid precursor protein and presenilin-1 mutations: lack of association with amyloid deposits. Behav Genet 1999;29:177–185.

Hong L, Koelsch G, Lin X, Wu S, Terzyan S, Ghosh AK, Zhang XC, Tang J. 2000. Structure of the protease domain of memapsin 2 (ß-secretase) complexed with inhibitor. Science 290:150–153.

Hoyer S. Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. Adv Exp Med Biol. 2004;541:135-52.

Hue L, Beauloye C, Bertrand L, Horman S, Krause U, Marsin AS, Meisse D, Vertommen D, Rider MH. New targets of AMP-activated protein kinase. Biochem Soc Trans. 2003 Feb;31(Pt 1):213-5.

Hwang DY, Cho JS, Lee SH, Chae KR, Lim HJ, Min SH, Seo SJ, Song YS, Song CW, Paik SG, Sheen YY, Kim YK. expressions of pathogenic phenotype in Alzheimer's diseased transgenic mice carrying NSE-controlled APPsw. Exp Neurol. 2004 Mar;186(1):20-32.

Imafuku I, Masaki T, Waragai M, Takeuchi S, Kawabata M, Hirai S, Ohno S, Nee LE, Lippa CF, Kanazawa I, Imagawa M, Okazawa H (1999) Presenilin 1 suppresses the function of c-Jun homodimers via interaction with QM/Jif-1. J Cell Biol 147:121–134.

Inomata, H., Nakamura, Y., Hayakawa, A., Takata, H., Suzuki, T., Miyazawa, K., Kitamura, N., 2003. A scaffold protein JIP-1b enhances amyloid precursor protein phosphorylation by JNK and its association with kinesin light chain 1. J. Biol. Chem. 278, 22946– 22955.

Inoshita S, Takeda K, Hatai T, Terada Y, Sano M, Hata J, Umezawa A, Ichijo H. Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. J Biol Chem. 2002 Nov 15;277(46):43730-4.

Irizarry MC, McNamara M, Fedorchak K, Hsiao K, Hyman BT: APPSw transgenic mice develop age-related Aß deposits and neuropil abnormalities, but no neuronal loss in CA1.Neuropathol Exp Neurol 1997;56:965–973.

Ivins KJ, Thornton PL, Rohn TT, Cotman CW. Neuronal apoptosis induced by beta-amyloid is mediated by caspase-8.Neurobiol Dis. 1999 Oct;6(5):440-9.

Jang JH, Aruoma OI, Jen LS, Chung HY, Surh YJ.Ergothioneine rescues PC12 cells from beta-amyloid-induced apoptotic death. Free Radic Biol Med. 2004 Feb 1;36(3):288-99.

Jarrett Jt, Berger Ep, And Lansbury Pt Jr. The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. Biochemistry 32: 4693–4697, 1993.

Kamal, A., Stokin, G.B., Yang, Z.H., Xia, C.H., Goldstein, L.S., 2000. Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron 28, 449– 459

Kamal A, Almenar-Queralt A, LeBlanc JF, Roberts EA, Goldstein LS. Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature. 2001 Dec 6; 414(6864): 643-8

Kanduc D, Mittelman A, Serpico, R. Cell death: apoptosis versus necrosis. Int J Oncol 2002;21:165-70.

Kang DE, Soriano S, Xia X, Eberhart CG, De Strooper B, Zheng H, Koo EH. Presenilin couples the paired phosphorylation of beta-catenin independent of axin: implications for beta-catenin activation in tumorigenesis. Cell. 2002 Sep 20;110(6):751-62.

Katoh I, Tomimori Y, Ikawa Y, Kurata SI.Dimerization and processing of procaspase-9 by redox stress in mitochondria. J Biol Chem. 2004 Jan 27 [Epub ahead of print]

Kaufmann, T., Schlipf, S., Sanz, J., Neubert, K., Stein, R., and Borner, C. 2003. Characterization of the signal that directs Bcl-xL, but not Bcl-2, to the mitochondrial outer membrane. J. Cell Biol. 160: 53–64.

Kaytor MD, Orr HT. The GSK3 beta signaling cascade and neurodegenerative disease. Curr Opin Neurobiol. 2002 Jun;12(3):275-8.

Kefas BA, Cai Y, Ling Z, Heimberg H, Hue L, Pipeleers D, Van de Casteele M. AMP-activated protein kinase can induce apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase. J Mol Endocrinol. 2003 Apr;30(2):151-61.

Kehoe PG, Russ C, McIlory S, Williams H, Holmans P, Holmes C, Liolitsa D, Vahidassr D, Passmore J, Lovestone S, Williams J, Owen MJ, Variation in DCP1; encoding ACE; is associated with susceptibility to Alzheimer disease, Nat. Genet. 21 (1999) 71–72.

Keil U, Bonert A, Marques CA, Scherping I, Weyermann J, Strosznajder J, Steiner B, Haass C, Müller WE, Eckert A (2004) "Involvement of NO in Aß induced mitochondrial dysfuction", *submitted*

Keller JN, Guo Q, Holtsberg FW, Bruce-Keller AJ, Mattson MP. 1998. Increased sensitivity to mitochondrial toxin-induced apoptosis in neural cells expressing mutant presenilin-1 is linked to perturbed calcium homeostasis and enhanced oxyradical production. J Neurosci 18:4439– 4450.

Kerr JF, Wyllie AH, Currie AR. Apoptosis:a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.

Kienlen-Campard P, Miolet S, Tasiaux B, Octave JN: Intracellular amyloid-ß 1–42, but not extracellular soluble amyloid-ß peptides, induces neuronal apoptosis. J Biol Chem 2002; 277:15666–15670.

Kim HS, Kim EM, Lee JP, Park CH, Kim S, Seo JH, Chang KA, Yu E, Jeong SJ, Chong YH, Suh YH. C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3beta expression. FASEB J. 2003 Oct; 17(13): 1951-3.

Kim JW, Chang TS, Lee JE, et al: Negative regulation of the SAPK/JNK signaling pathway by presenilin 1. J Cell Biol 2001;153: 457–463.

Kim TW, Pettingell WH, Jung YK, Kovacs DM, Tanzi RE. Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science. 1997 Jul 18; 277(5324): 373-6.

Kimberly, W. T., Xia, W., Rahmati, R., Wolfe, M. S. and Selkoe, D. J. (2000). The transmembrane aspartates in presenilin 1 and 2 are obligatory for g-secretase activity and amyloid ß-protein generation. J. Biol. Chem. 275, 3173-3178.

Kinoshita A, Whelan CM, Smith CJ, Berezovska O, Hyman BT. Direct visualization of the gamma secretase-generated carboxyl-terminal domain of the amyloid precursor protein: association with Fe65 and translocation to the nucleus. J Neurochem. 2002 Aug; 82(4): 839-47.

Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT. Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. J Cell Sci. 2003 Aug 15;116(Pt 16):3339-46. Epub 2003 Jun 26.

Kirsch C, Eckert GP, Müller WE (2003) Statin effects on cholesterol micro-domains in brain plasma membranes. Biochem Pharmacol 65: 843-856

Kirsch C, Eckert GP, Müller WE (2002) Cholesterol attenuates the membrane-perturbing properties of beta-amyloid peptides. Amyloid 9: 149-159

Kirschenbaum F, Hsu SC, Cordell B, McCarthy JV. Glycogen synthase kinase 3-beta regulates presenilin 1 C-terminal fragment levels. J Biol Chem 2001;276:30701–30707.

Kounnas MZ, Moir RD, Rebeck GW, Bush AI, Argraves WS, Tanzi RE, Hyman BT, Strickland DK, LDL receptor-related protein; a multifunctional ApoE receptor; binds secreted beta-amyloidprecursor protein and mediates its degradation, Cell. 82 (1995)331–340.

Krohn A.J., Preis E., Prehn J.H., Staurosporine-induced apoptosis of cultured rat hippocampal neurons involves capase-1-like proteases as upstream initiators and increased production of superoxide as a main downstream effector, J. Neurosci., Volume: 18, (1998), pp. 8186-8197

Kumar S and Vaux DL (2002) Apoptosis. A cinderella caspase takes center stage. Science 297: 1290–1291

Kuo YM, Emmerling MR, Vigo-Pelfrey C, Kasunic TC, Kirkpatrick JB, Murdoch GH, Ball MJ, Roher AE. Water-soluble Abeta (N-40, N-42) oligomers in normal and Alzheimer disease brains. J Biol Chem. 1996 Feb 23; 271(8): 4077-81.

LaFerla FM, Tinkle BT, Bieberich CJ, Haudenschild CC, Jay G. The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. Nat Genet 1995;9:21–30.

Larsen AK, Moller MT, Blankson H, Samari HR, Holden L, Seglen PO. Naringin-sensitive phosphorylation of plectin, a cytoskeletal cross-linking protein, in isolated rat hepatocytes. J Biol Chem. 2002 Sep 20;277(38):34826-35.

Lassus P, Opitz-Araya X and Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297: 1352–1354

Law A, Gauthier S, Quirion R.Say NO to Alzheimer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. Brain Res Brain Res Rev. 2001 Mar;35(1):73-96.

Law A, O'Donnell J, Gauthier S, Quirion R.Neuronal and inducible nitric oxide synthase expressions and activities in the hippocampi and cortices of young adult, aged cognitively unimpaired, and impaired Long-Evans rats.Neuroscience. 2002;112(2):267-75.

Lee MS, Kao SC, Lemere CA, Xia W, Tseng HC, Zhou Y, Neve R, Ahlijanian MK, Tsai LH. APP processing is regulated by cytoplasmic phosphorylation. J Cell Biol. 2003 Oct 13;163(1):83-95.

Lee,S.F.; Shah,S.; Li,H.; Yu,C.; Han,W.; Yu,G. Mammalian APH-1 interacts with presenilin and nicastrin, and is required for intramembrane proteolysis of APP and Notch(2002) *J.Biol.Chem.* **277,** 45013-45019

Leutner S, Eckert A, Muller WE. ROS generation, lipid peroxidation and antioxidant enzyme activities in the aging brain. J Neural Transm. 2001; 108(8-9): 955-6

Leutz S, Steiner B, Marques CA, Haass C, Muller WE, Eckert A. Reduction of trophic support enhances apoptosis in PC12 cells expressing Alzheimer's APP mutation and sensitizes cells to staurosporine-induced cell death. J Mol Neurosci. 2002 Jun;18(3):189-201.

Li R, Yang L, Lindholm K, Konishi Y, Yue X, Hampel H, Zhang D, Shen Y. Tumor necrosis factor death receptor signaling cascade is required for amyloid-beta protein-induced neuron death. J Neurosci. 2004 Feb 18;24(7):1760-71

Linnik MD, Zobrist RH, Hatfield MD. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 1993;24:2002-9.

Loetscher H, Deuschle U, Brockhaus M, Reinhardt D, Nelboeck P, Mous J, Grunberg J, Haass C, Jacobsen H. Presenilins are processed by caspase-type proteases. J Biol Chem. 1997 Aug 15;272(33):20655-9.

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW. Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci USA 1993;90:7951–5.

Lotem J, Peled-Kamar M, Groner Y, Sachs L.Cellular oxidative stress and the control of apoptosis by wild-type p53, cytotoxic compounds, and cytokines. Proc Natl Acad Sci U S A. 1996 Aug 20;93(17):9166-71.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Lu D. C., Rabizadeh S., Chandra S., Shayya R. F., Ellerby L. M. YeX., Salvesen G. S., Koo E. H. and Bredesen D. E. (2000) A second cytotoxic proteolytic peptide derived from amyloid b-protein precursor. Nat. Med. 6, 397–404.

Lu DC, Shaked GM, Masliah E, Bredesen DE, Koo EH. Amyloid beta protein toxicity mediated by the formation of amyloid-beta protein precursor complexes. Ann Neurol. 2003 Dec; 54(6): 781-9.

Lu DC, Soriano S, Bredesen DE, Koo EH. Caspase cleavage of the amyloid precursor protein modulates amyloid beta-protein toxicity. J Neurochem. 2003bNov;87(3):733-41.

Lue LF, Kuo YM, Roher AE, Brachova L, Shen Y, Sue L, Beach T, Kurth JH, Rydel RE, Rogers J. Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am J Pathol. 1999 Sep; 155(3): 853-62.

Luo Y, Bolon B, Kahn S, Bennet BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R. 2001. Mice de.cient in BACE 1, the Alzheimer's y-secretase, have normal phenotype and abolished ß-amyloid generation. Nat Neurosci 4:231–232.

Madesh M, Hajnoczky G. VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome c release. J Cell Biol. 2001 Dec 10;155(6):1003-15.

Mahley R.W., Rall S.C., Apolipoprotein E: far more than a lipid transport protein, Ann. Rev. Genomics Hum. Genet. 1 (2000) 507–537.

Mancini M, Machamer CE, Roy S, Nicholson DW, Thornberry NA, Casciola-Rosen LA, Rosen A. Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. J Cell Biol. 2000 May 1;149(3):603-12.

Mandelkow E. Alzheimer's disease. The tangled tale of tau. Nature. 1999 Dec 9;402(6762):588-9.

Mann D.M. The pathological association between Down syndrome and Alzheimer's disease, Mech. Age Dev. 43 (1985) 99–136.

Maroto R, Perez-Polo JR .BCL-2-related protein expression in apoptosis: oxidative stress versus serum deprivation in PC12 cells. J Neurochem. 1997 Aug;69(2):514-23.

Marques CA, Eckert A, Steiner B, Haass C, Müller WE. Activation of caspase-3 by the Swedish amyloid precursor protein mutation 42. Frühjahrstagung der DGPT, Mainz, Germany, 2001 N-S ARCH PHARMACOL 363 (4): 348 Suppl. S APR 2001

Marques CA, Keil U, Bonert A, Steiner B, Haass C, Muller WE, Eckert A. Neurotoxic mechanisms caused by the Alzheimer's disease-linked Swedish amyloid precursor protein mutation: oxidative stress, caspases, and the JNK pathway. J Biol Chem. 2003 Jul 25;278(30):28294-302

Martin JB. Molecular basis of the neurodegenerative disorders. N Engl J Med 1999; 340:1970-80.

Martin LJ. Neuronal cell death in nervous system development, disease, and injury. Int J Mol Med 2001;7:455-78

Massieu L, Moran J, Christen Y.Effect of Ginkgo biloba (EGb 761) on staurosporine-induced neuronal death and caspase activity in cortical cultured neurons. Brain Res. 2004 Mar 26;1002(1-2):76-85.

Matsuda S, Yasukawa T, Homma Y, et al: c- Jun N-terminal kinase (JNK)-interacting protein-1b/islet-brain-1 scaffolds Alzheimer's amyloid precursor protein with JNK. J Neurosci 2001;21:6597–6607.

Matsuda, S., Matsuda, Y., Dadamio, L., 2003. Amyloid beta protein precursor, but not APLP2, is bridged to the kinesin light chain by the scaffold protein JNK-interacting protein 1. J. Biol. Chem. 278, 38601– 38606.

Mattson MP, Bruce AJ, Mark RJ. Amyloid cytotoxicity and Alzheimer's disease: roles of membrane oxidation and perturbed ion homeostasis. In: Brioni JD, ed. Pharmacological treatment of Alzheimer's disease: molecular and neurobiological foundations. New York: Wiley-Liss, 1997: 239–85.

Mattson MP, Partin J, Begley JG. Amyloid beta-peptide induces apoptosis-related events in synapses and dendrites. Brain Res 1998;807:167–76.

Mattson MP, Liu D. Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders. Neuromol Med 2002;215–31.

Mattson MP, Sic L. Calcium orchestrates apoptosis Nature Cell Biology Dez 2003 5, 1041 - 1043

Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S. Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. J Biol Chem. 1997 Oct 3;272(40):25238-42.

Maurer I, Zierz S, Moller HJ.A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiol Aging. 2000 May-Jun;21(3):455-62.

McLean CA, Cherny RA, Fraser FW, Fuller SJ, Smith MJ, Beyreuther K, Bush AI, Masters CL. Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann Neurol. 1999 Dec; 46(6): 860

Mecocci P, MacGarvey U, Beal MF, Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease, Ann. Neurol. 36 (1994) 747–751.

Mesner PW, Epting CL, Hegarty JL, Green SH. A timetable of events during programmed cell death induced by trophic factor withdrawal from neuronal PC12 cells. J Neurosci. 1995 Nov;15(11):7357-66.

Mielke K, Herdegen T. JNK and p38 stresskinases--degenerative effectors of signal-transduction-cascades in the nervous system. Prog Neurobiol. 2000 May;61(1):45-60.

Mikolajczyk M, Shi J, Vaillancourt RR, Sachs NA, Nelson M. The cyclin-dependent kinase 11(p46) isoform interacts with RanBPM. Biochem Biophys Res Commun. 2003 Oct 10;310(1):14-8.

Misonou H, Morishima-Kawashima M, Ihara Y. Oxidative stress induces intracellular accumulation of amyloid beta-protein (Abeta) in human neuroblastoma cells. Biochemistry 2000;39:6951–9.

Mitchell P, Chemiosmotic coupling in oxidative and photosynthetic phosphorylation, Biol. Rev. 41 (1996) 445–502.

Mizuno T, Nakata M, Naiki H, Michikawa M, Wang R, Haass C, Yanagisawa K [1999]. Cholesterol-dependent generation of a seeding amyloid beta-protein in cell culture. J Biol Chem., 274(21): 15110-15114.

Moechars D, Lorent K, De Strooper B, Dewachter I, Van Leuven F: Expression in brain of amyloid precursor protein mutated in the ·α-secretase site causes disturbed behavior, neuronal degeneration and premature death in transgenic mice. EMBO J 1996;15:1265–1274.

Morfini G, Pigino G, Beffert U, Busciglio J, Brady ST (2002) Fast axonal ransport misregulation and Alzheimer's disease. Neuromolecular Med 2:89–100.

Morfini G, Szebenyi G, Elluru R, Ratner N, Brady ST (2002b) Glycogen synthase kinase-3 phosphorylates kinesin light chains and negatively regulates kinesin-based motility. EMBO J 21:281–293.

Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y, Greenberg ME. Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. J Neurosci. 2001 Oct 1; 21(19): 7551-60.

Mosmann, T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods1983, 65, 55–63.

Müller WE, Kirsch C, Eckert GP (2001) Membrane disordering effects of beta-amyloid peptides. Biochem Soc Trans 29: 617-623

Mumm JS, Kopan R: Notch signaling: from the outside in. Dev Biol 2000, 228:151-165. Myers AJ, Goate AM. The genetics of late-onset Alzheimer's disease. Curr Opin Neurol 2001;14:433-40.

Nathan BP, Bellosta S, Sanan DA, Weisgraber KH, Mahley RW, Pitas RE [1994]. Differential effects of apolipoproteins E3 and E4 on neuronal growth in vitro. Science, 264(5160): 850-852.

Narain Y, Yip A, Murphy T, Brayne C, Easton D, Evans JG, Xuereb J, Cairns N, Esiri MM, Furlong RA, Rubinsztein DC, The ACE gene and Alzheimer's disease susceptibility, J. Med. Genet.37 (2000) 695–697.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry.
J Immunol Methods. 1991 Jun 3;139(2):271-9.

Nishimoto I., Okamoto, Y. Matsuura, S. Takahashi, T. Okamoto, Y. Murayama, E. Ogata, Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G(o), Nature 362 (1993) 75–79.

Nishimura I., Uetsuki T., Kuwako K., Hara T., Kawakami T., Aimoto S. and Yoshikawa K. (2002) Cell death induced by a caspase-cleaved transmembrane fragment of the Alzheimer amyloid precursor protein. Cell Death Differen. 9, 199–208.

G . Olivieri, C. Hess, E. Savaskan, C. Ly, F. Meier, G. Baysang, M. Brockhaus, F. Muller-Spahn, Melatonin protects SHSY5Y neuroblastoma cells from cobalt-induced oxidative stress, neurotoxicity and increased beta-amyloid secretion, J. Pineal Res. 31 (2001) 320–325.

Pantano C, Shrivastava P, McElhinney B, Janssen-Heininger Y. Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-Jun N-terminal kinase. J Biol Chem. 2003 Nov 7;278(45):44091-6.

Parkinson Study Group. safety and tolerability of a mixed lineage kinase inhibitor (CEP-1347) in PD. Neurology. 2004 Jan 27;62(2):330-2.

Parola, M.; Robino, G.; Marra, F.; Pinzani, M.; Bellomo, G.; Leonarduzzi, G.; Chiarugi, P.;Camandola, S.; Poli, G.; Waeg, G.; Gentilini, P.; Dianzani, M. U. HNE interacts directly with JNK isoforms in human hepatic stellate cells. *J. Clin. Invest.* **102:** 1942–1950; 1998.

Paroni G, Henderson C, Schneider C and Brancolini C (2002) Caspase-2 can trigger cytochrome C release and apoptosis from the nucleus. J. Biol. Chem. 277: 15147–15161

Parshad RP, Sanford KK, Price FM, Melnick LK, Nee LE, Schapiro MB, Tarone RE, Robbins JH. Fluorescent light-induced chromatid breaks distinguish Alzheimer disease cells from normal cells in tissue culture. Proc Natl Acad Sci U S A. 1996 May 14;93(10):5146-50.

Pei JJ, Braak E, Braak H, Grundke-Iqbal I, Iqbal K, Winbald B, Cowburn RF. Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. *J Alzheimers Dis* 3:41–48, 2001.

Pellegrini L, Passer BJ, Tabaton M, Ganjei JK, D'Adamio L. Alternative, non-secretase processing of Alzheimer's beta-amyloid precursor protein during apoptosis by caspase-6 and -8. J Biol Chem. 1999 Jul 23;274(30):21011-6.

Pereira C, Santos MS, Oliveira C. Involvement of oxidative stress on the impairment of energy metabolism induced by A beta peptides on PC12 cells: protection by antioxidants. Neurobiol Dis. 1999 Jun;6(3):209-19.

Perez RG, Zheng H, Van Der Ploeg Lh, Koo EH. The beta amyloid precursor protein of Alzheimer's disease enhances neuron viability and modulates neuronal polarity. J Neurosci 17: 9407–9414, 1997.

Perry G, Nunomura A, Raina AK, Aliev G, Siedlak SL, Harris PL, Casadesus G, Petersen RB, Bligh-Glover W, Balraj E, Petot GJ, Smith MA. A metabolic basis for Alzheimer disease. Neurochem Res. 2003 Oct; 28(10): 1549-52.

Phiel CJ, Wilson CA, Lee VM, Klein PS. GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. Nature. 2003 May 22; 423(6938): 435-9.

Pias EK, Aw TY. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. Cell Death Differ. 2002 Sep;9(9):1007-16.

Pigino G, Morfini G, Pelsman A, Mattson MP, Brady ST, Busciglio J. Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. J Neurosci. 2003 Jun 1; 23(11): 4499-508.

Pitschke M, Prior R, Haupt M, Riesner D. Detection of single amyloid beta-protein aggregates in the cerebrospinal fluid of Alzheimer's patients by fluorescence correlation spectroscopy. Nat Med. 1998 Jul; 4(7): 832-4.

Planel E, Yasutake K, Fujita SC, Ishiguro K: Inhibition of protein phosphatase 2A overrides tau protein kinase I/glycogen synthase kinase 3 beta and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse. J Biol Chem 2001;276:34298–34306.

Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. Free Radic Biol Med. 2002 Dec 1;33(11):1451-64

Read SH, Baliga BC, Ekert PG, Vaux DL, Kumar S. A novel Apaf-1-independent putative caspase-2 activation complex.J Cell Biol. 2002 Dec 9;159(5):739-45.

G.W. Rebeck, S.D. Harr, D.K. Strickland, B.T. Hyman, Multiple, diverse senile plaque-associated proteins are ligands of an apolipoprotein E receptor, the alpha 2-macroglobulin receptor/lowdensity-lipoprotein receptor-related protein, Ann. Neurol. 37 (1995) 211–217.

Reveneau S, Paumelle R, Deheuninck J, Leroy C, De Launoit Y, Fafeur V. Inhibition of JNK by HGF/SF prevents apoptosis induced by TNF-alpha. Ann N Y Acad Sci. 2003 Dec;1010:100-3.

Reynolds CH, Utton MA, Gibb GM, Yates A, Anderton BH.Stress-activated protein kinase/c-jun N-terminal kinase phosphorylates tau protein. J Neurochem. 1997 Apr;68(4):1736-44.

Robertson JD, Enoksson M, Suomela M, Zhivotovsky B and Orrenius S (2002) Caspase-2 acts upstream of mitochondria to promote cytochrome c release during etoposide-induced apoptosis. J. Biol. Chem. 277: 29803–29809

Rohn TT, Ivins KJ, Bahr BA, et al. A monoclonal antibody to amyloid precursor protein induces neuronal apoptosis. J Neurochem 2000;74:2331–2342.

Rottkamp CA, Nunomura A, Raina AK, Sayre LM, Perry G, Smith MA. Oxidative stress, antioxidants, and Alzheimer disease. Alzheimer Dis Assoc Disord. 2000;14 Suppl 1:S62-6.

Ryder J, Su Y, Ni B.Akt/GSK3beta serine/threonine kinases: evidence for a signalling pathway mediated by familial Alzheimer's disease mutations. Cell Signal. 2004 Feb;16(2):187-200.

Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM, Zon LI. Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun.Nature. 1994 Dec 22-29;372(6508):794-8.

Sang H, Lu Z, Li Y, Ru B, Wang W, Chen J. Phosphorylation of tau by glycogen synthase kinase 3beta in intact mammalian cells influence the stability of microtubules. Neurosci Lett 2001;312:141–144.

Sani S, Traul D, Klink A, Niaraki N, Gonzalo-Ruiz A, Wu CK, Geula C (2003) Distribution, progression and chemical composition of cortical amyloid-beta deposits in aged rhesus monkeys: similarities to the human. Acta Neuropathol (Berl) 105: 145-156

Saunders AM, Schmader K, Breitner JCS et al. (1993) Apoplipoprotein E4 allele distributions in late-onset Alzheimer´s disease and in other amyloid-forming diseases. Lancet 342: 710-711

Savage MJ, Lin YG, Ciallella JR, Flood DG, Scott RW. Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. J Neurosci. 2002 May 1; 22(9): 3376-85.

Scheinfeld MH, Roncarati R, Vito P, Lopez PA, Abdallah M, D'Adamio L: Jun NH2-terminal kinase (JNK) interacting protein 1 (JIP1) binds the cytoplasmic domain of the Alzheimer's beta-amyloid precursor protein (APP). J Biol Chem 2002;277:3767–3775.

Schindowski K, Frohlich L, Maurer K, Muller WE, Eckert A. Age-related impairment of human T lymphocytes' activation: specific differences between CD4(+) and CD8(+) subsets. Mech Ageing Dev. 2002 Feb;123(4):375-90.

Schindowski K, Kratzsch T, Peters J, Steiner B, Leutner S, Touchet N, Maurer K, Czech C, Pradier L, Frolich L, Muller WE, Eckert A.Impact of aging: sporadic, and genetic risk factors on vulnerability to apoptosis in Alzheimer's disease. Neuromolecular Med. 2003;4(3):161-78.

Schroeter EH, Kisslinger JA, Kopan R. 1998 Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. Nature 393:382–86

Schwabe RF, Uchinami H, Qian T, Bennett BL, Lemasters JJ, Brenner DA. Differential requirement for c-Jun NH2-terminal kinase in TNFalpha- and Fas-mediated apoptosis in hepatocytes. FASEB J. 2004 Apr;18(6):720-2.

Schweizer A, Briand C, Grutter MG., Crystal structure of caspase-2, apical initiator of the intrinsic apoptotic pathway. J Biol Chem. 2003 Oct 24;278(43):42441-7

Selkoe D.J. Selective ectodomain phosphorylation and regulated cleavage of b-amyloid precursor protein. EMBO J 13: 534–542, 1994.

Selkoe DJ. Normal and abnormal biology of the beta-amyloid precursor protein. Annu Rev Neurosci. 1994;17:489-517.

Selkoe,D.J. Amyloid beta-protein and the genetics of Alzheimer's disease (1996) *J.Biol.Chem.* **271,** 18295-18298

Selkoe, D J (2001). Clearing the brain's amyloid cobwebs. Neuron, 32, 177–180.

Selkoe, DJ (2002) Alzheimer's disease is a synaptic failure. Science 298, 789–791

Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G. and Castro, J. L. (2000). L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid beta-protein precursor gamma-secretase activity. Biochemistry 39, 8698-8704.

Shen J, Bronson RT, Chen DF, Xia WM, Selkoe DJ, Tonegawa S. 1997. Skeletal and CNS defects in presenilin-1 deficient mice. Cell 89:629–39

Shearwin-Whyatt LM, Harvey NL, Kumar S. Subcellular localization and CARD-dependent oligomerization of the death adaptor RAIDD. Cell Death Differ. 2000 Feb; 7(2): 155-65.

Shigematsu T, Priori ES, Dmochowski L, Wilbur JR Immunoelectron microscopic studies of type C virus particles in ESP-1 and HEK-1-HRLV cell lines. Nature 1971. 1971 234: 412-414

Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. Nature. 1999 Jun 3;399(6735):483-7. Erratum in: Nature 2000 Oct 12;407(6805):767.

Shoji M, Iwakami N, Takeuchi S, Waragai M, Suzuki M, Kanazawa I, Lippa CF, Ono S, Okazawa H. JNK activation is associated with intracellular ß-amyloid accumulation. *Brain Res Mol Brain Res* 85:221–233, 2000.

Sisodia SS. Biomedicine. A cargo receptor mystery APParently solved? Science. 2002 Feb 1;295(5556):805-7

Slunt Hh, Thinakaran G, Von Koch C, Lo Acy, Tanzi Re, Sisodia S. Expression of a ubiquitous, cross-reactive homologue of the mouse b-amyloid precursor protein (APP). J Biol Chem 269:2637–2644, 1994.

Smith MA, Hirai K, Hsiao K, Pappolla MA, Harris PLR, Siedlak SL, Tabaton M, and Perry G. Amyloid-b deposition in Alzheimer transgenic mice is associated with oxidative stress. *J Neurochem*70: 2212–2215, 1998.

Smith MA , Nunomura A, Zhu X, Takeda A, Perry G, Metabolic, metallic, and mitotic sources of oxidative stress in Alzheimer disease, Antioxid. Redox Signal. 2 (2000) 413–420.

Sprick MR, Rieser E, Stahl H, Grosse-Wilde A, Weigand MA and Walczak H. (2002). Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. EMBO J., 21, 4520–4530.

Standen CL, Brownlees J, Grierson AJ, et al. Phosphorylation of thr(668) in the cytoplasmic domain of the Alzheimer's disease amyloid precursor protein by stress-activated protein kinase 1b (Jun N-terminal kinase-3). J Neurochem 2001;76:316–320.

Steiner H, Capell A, Pesold B, Citron M, Kloetzel PM, Selkoe DJ, Romig H, Mendla K, Haass C. Expression of Alzheimer's disease-associated presenilin-1 is controlled by proteolytic degradation and complex formation. J Biol Chem. 1998 Nov 27;273(48):32322-31.

Steiner,H.; Winkler,E.; Edbauer,D.; Prokop,S.; Basset,G.; Yamasaki,A.; Kostka,M.; Haass,C. PEN-2 is an integral component of the gamma -secretase complex required for coordinated expression of presenilin and nicastrin(2002) *J.Biol.Chem.* **277,** 39062-39065

St George-Hyslop PH, McLachlan DC, Tsuda T (1994) Alzheimer´s disease and possible gene interaction. Science 263:537

Strittmatter WJ, Weisgraber KH, Goedert M, Saunders AM, Huang D, Corder EH, Dong LM, Jakes R, Alberts MJ, Gilbert JR, Hulette C, Einstein G, Schmechel DE, Pericak-Vance MA, Roses AD, Hypothesis: microtubule instability and paired helical filament formation in the Alzheimer disease brain are related to apolipoprotein E genotype, Exp. Neurol. 125 (1994) 163–171.

Su JH, Anderson AJ, Cummings BJ, Cotman CW. Immunohistochemical evidence for apoptosis in Alzheimer's disease. Neuroreport. 1994 Dec 20; 5(18): 2529-33.

Su JH, Anderson AJ, Cribbs DH, Tu C, Tong L, Kesslack P, Cotman CW. Fas and Fas ligand are associated with neuritic degeneration in the AD brain and participate in beta-amyloid-induced neuronal death. Neurobiol Dis. 2003 Apr;12(3):182-93.

Sudo H, Jiang H, Yasukawa T, et al. Antibody regulated neurotoxic function of cell-surface _-amyloid precursor protein. Mol Cell Neurosci 2000;16:708 –723.

Suo Z, Fang C, Crawford F, Mullan M. Superoxide free radical intracellular calcium mediate Aß1–42 induced endothelial toxicity.Brain Res 1997;762:144–52.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M., Kroemer, G., 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397, 441–446.

Suzuki,N.; Cheung,T.T.; Cai,X.D.; Odaka,A.; Otvos,L.,Jr.; Eckman,C.; Golde,T.E.; Younkin,S.G. An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants (1994) *Science* **264,** 1336-1340

Suzaki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, Tamaki T.Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. J Biol Chem. 2002 Mar 15; 277(11)

Suzuki Y, Ono Y, Hirabayashi Y. Rapid and specific reactive oxygen species generation via NADPH oxidase activation during fas-mediated apoptosis, FEBS Lett. 425 (1998) 209–212.

Swannie HC, Kaye SB Protein kinase C inhibitors. Curr Oncol Rep.2000 Jan;4(1):37-46. Tabira T, Chui DH, Kuroda S: Significance intracellular Aß42 accumulation in Alzheimer's disease. Front Biosci 2002;7:a44–a49.

Tabner BJ, Turnbull S, El-Agnaf OM, Allsop D. Formation of hydrogen peroxide and hydroxyl radicals from A(beta) and alpha-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease. Free Radic Biol Med. 2002 Jun 1;32(11):1076-83.

Takahashi A, Masuda A, Sun M, Centonze VE, Herman B.Oxidative stress-induced apoptosis is associated with alterations in mitochondrial caspase activity and Bcl-2-dependent alterations in mitochondrial pH (pH(m)). Brain Res Bull. 2004 Feb 15;62(6):497-504.

Takeda K, Araki W, Tabira T. Enhanced generation of intracellular Abeta42 amyloid peptide by mutation of presenilins PS1 and PS2. Eur J Neurosci. 2004 Jan; 19(2): 258-364.

Tamaoki T, Nomoto H, Takahashi I et al. Staurosporine, potent inhibitor of phospholipid/Ca++ dependent protein kinase. Biochem Biophys Res Commun 1986;135:397-402.

Tamagno E, Bardini P, Obbili A, Vitali A, Borghi R, Zaccheo D, Pronzato MA, Danni O, Smith MA, Perry G, Tabaton M.Oxidative stress increases expression and activity of BACE in NT2 neurons. Neurobiol Dis. 2002 Aug;10(3):279-88.

Tandon, A., Rogaeva, E. A., Mullan, M., and St. George-Hyslop, P. (2000) Molecular genetics of Alzheimer's disease: the role of beta-amyloid and the presenilins. Curr. Opin. Neurol. 13, 377–384.

Tanii H, Ankarcrona M, Flood F, Nilsberth C, Mehta ND, Perez-Tur J, Winblad B, Benedikz E, Cowburn RF. 2000. Alzheimer's disease presenilin-1 exon 9 deletion and L250S mutations sensitize SH-SY5Y neuroblastoma cells to hyperosmotic stress-induced apoptosis. Neuroscience 95:593– 601.

Tanzi R.E, Gusella, P.C. Watkins, G.A. Bruns, P. St George-Hyslop, M.L. Van Keuren, D. Patterson, S. Pagan, D.M. Kurnit, R.L. Amyloid beta protein gene: cDNA mRNA distribution and genetic linkage near the Alzheimer locus, Science 235 1987 880–884.

Tanzi,R.E.; Kovacs,D.M.; Kim,T.W.; Moir,R.D.; Guenette,S.Y.; Wasco,W. The gene defects responsible for familial Alzheimer's disease (1996) *Neurobiol.Dis.* **3,** 159-168

Tanzi, R. E., Bertram, L. (2001). New frontiers in Alzheimer's disease genetics. Neuron, 32, 181–184.

Taru H, Kirino Y, Suzuki T: Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. J Biol Chem 2002a;277:27567–27574.

Taru H, Iijima K, Hase M, Kirino Y, Yagi Y, Suzuki T: Interaction of Alzheimer's beta amyloid precursor family proteins with scaffold proteins of the JNK signaling cascade. J Biol Chem 2002b;277:20070–20078.

Taru H, Suzuki T. Facilitation of stress-induced phosphorylation of beta -amyloid precursor protein family members by X11-like/Mint2 protein. J Biol Chem. 2004 Feb 17 [Epub ahead of print]

Terro F, Czech C, Esclaire F, Elyaman W, Yardin C, Baclet MC, Touchet N, Tremp G, Pradier L, Hugon J. Neurons overexpressing mutant presenilin-1 are more sensitive to apoptosis induced by endoplasmic reticulum-Golgi stress. J Neurosci Res. 2002 Aug 15; 69(4): 530-9.

Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, et al. 1996. Endoprotreolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron 17:181–90

Thomas LB, Gates DJ, Richfield EK, O'Brien TF, Schweitzer JB, Steindler DA. DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. Exp Neurol 1995;133:265-72.

Thornberry NA, Lazebnik Y (1998) Caspases: Enemies within- Science 281: 1312-1316

Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol. 2003 May; 4(5): 404-9.

Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K, Shelanski ML. Caspase-2 mediates neuronal cell death induced by beta-amyloid. J Neurosci. 2000 Feb 15;20(4):1386-92.

Troy,C.M.; Rabacchi,S.A.; Hohl,J.B.; Angelastro,J.M.; Greene,L.A.; Shelanski,M.L. Death in the balance: alternative participation of the caspase-2 and -9 pathways in neuronal death induced by nerve growth factor deprivation (2001) *J.Neurosci.* **21,** 5007-5016

Troy,C.M.; Rabacchi,S.A.; Xu,Z.; Maroney,A.C.; Connors,T.J.; Shelanski,M.L.; Greene,L.A. beta-Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activationJ Neurochem. 2001 Apr;77(1):157-64.

Tsujimoto V, Shimizu S, Bcl-2 family: life-or-or death switch, FEBS Lett. 466 (2000) 6–10.

Uchida, K.; Shiraishi, M.; Naito, Y.; Torii, Y.; Nakamura, Y.; Osawa, T. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *J. Biol. Chem.* **274:** 2234–2242; 1999.

Vancea J.E., Campenot R.B., Vancec D.E., The synthesis and transport of lipids for axonal growth and nerve regeneration, Biochim. Biophys. Acta 1486 (2000) 84–96.

Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiaz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, Science 286, 735-741

Vekrellis K, Ye Z, Qiu WQ Walsh D, Hartley D Chesneau V. Rosner MR, Selkoe DJ, Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme, J. Neurosci. 20 (2000) 1657–1665.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J., and Vaux, D.L. 2000. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102: 43–53.

Verhey KJ, Rapoport TA. Kinesin carries the signal. Trends Biochem Sci. 2001 Sep; 26(9): 545-50

Verhey KJ, Meyer D, Deehan R, Blenis J, Schnapp BJ, Rapoport TA, Margolis B. Cargo of kinesin identified as JIP scaffolding proteins and associated signalling molecules. J Cell Biol. 2001 Mar 5; 152(5): 959-70.

Verpillat PBouley S, Campion D, Hannequin D, Dubois B, Belliard S, Puel M, Thomas-Anterion C, Agid Y, Brice A, Clerget-Darpoux F, Use of haplotype information to test involvement of the LRP gene in Alzheimer's disease in the French population, Eur. J. Hum. Genet. 9 (2001) 464–468.

Vezina J, Tschopp C, Andersen E, Muller K. Overexpression of a C-terminal fragment of presenilin 1 delays anti-Fas induced apoptosis in Jurkat cells. Neurosci Lett. 1999 Mar 19;263(1):65-8.

Vieira HL, Haouzi D, El Hamel C, Jacotot E, Belzacq AS, Brenner C, Kroemer G. Permeabilization of the mitochondrial inner membrane during apoptosis: impact of the adenine nucleotide translocator. Cell Death Differ. 2000 Dec;7(12):1146-54

Villa A., Latasa M.J. , Pascual A., Nerve growth factor modulates the expression and secretion of beta-amyloid precursor protein through different mechanisms in PC12 cells, J. Neurochem. 77 (2001) 1077–1084.

Vito P, Ghayur T, D'Adamio L. Generation of anti-apoptotic presenilin-2 polypeptides by alternative transcription, proteolysis, and caspase-3 cleavage. J Biol Chem. 1997 Nov 7;272(45):28315-20.

Vivo C, Liu W, Broaddus VC. c-Jun N-terminal kinase contributes to apoptotic synergy induced by tumor necrosis factor-related apoptosis-inducing ligand plus DNA damage in chemoresistant, p53 inactive mesothelioma cells. J Biol Chem. 2003 Jul 11;278(28):25461-7.

Vocero-Akbani AM, Heyden NV, Lissy NA, Ratner L and Dowdy SF (1999) Killing HIV-infected cells by transduction with an HIV protease- activated caspase-3 protein [see comments]. Nat. Med. 5: 29 ± 33

Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature. 2002 Apr 4; 416(6880): 535-9.

Walter J, Capell A, Hung Ay, Langen H, Schnolzer M, Thinkaran Sisodia S, Selkoe DJ, And Haass C. Ectodomain phosphorylation of b-amyloid precursor protein at two distinct cellular locations. J Biol Chem 272: 1896–1903, 1997.

Wang X, Yang C, Chai J, Shi Y, Xue D. Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. Science. 2002 Nov 22; 298(5598): 1587-92.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 2001 Apr 27;292(5517):727-30.

Weihl CC, Ghadge GD, Kennedy SG, Hay N, Miller RJ, Roos RP.Mutant presenilin-1 induces apoptosis and downregulates Akt/PKB. J Neurosci. 1999 Jul 1;19(13):5360-9.

Wellington CL, Hayden MR. Caspases and neurodegeneration: on the cutting edge of new therapeutic approaches. Clin Genet. 2000 Jan; 57(1): 1-10.

Wilson CA, Doms RW, Lee VM. Distinct presenilin-dependent and presenilin-independent gamma-secretases are responsible for total cellular Abeta production. J Neurosci Res. 2003 Nov 1; 74(3): 361-9.

Wirths O, Multhaup G, Czech C, Blanchard V, Moussaoui S, Tremp G, Intraneuronal Abeta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double transgenic mice. Neurosci Lett 2001;306:116–20.

Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T. And Selkoe, D. J. (1999a). Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and g-secretase activity. Nature 398, 513-517.

Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B. L., Rahmati, T., Donkor, I. O. and Selkoe, D. J. (1999b). Peptidomimetic probes and molecular modeling suggest Alzheimer's g-secretase is an intramembrane-cleaving aspartyl protease. Biochemistry 38, 4720-4727.

Wolozin B, Alexander P, Palacino J (1998) Regulation of apoptosis by presenilin 1. Neurobiol Aging 19:S23–S27.

Wolozin B, Iwasaki K, Vito P. Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. Science 1996: 274: 1710–1713.

Wong PC, Zhen H, Chen H, Becher MW, Sirinathsinghji DJS, et al. 1997. Presenilin 1 is required for Notch 1 and D111 expression in the paraxial mesoderm. Nature 397:288–92

J.W. Wright, J.W. Harding, Regulatory role of brain angiotensins in the control of physiological and behavioral responses, Brain Res. Brain Res. Rev. 17 (1992) 227–262.

Xia, X., Qian, S., Soriano, S., Wu, Y., Fletcher, A. M., Wang, X. J., Koo, E. H., Wu, X. and Zheng, H. (2001). Loss of presenilin 1 is associated with enhanced beta-catenin signaling and skin tumorigenesis. Proc. Natl. Acad.Sci. USA 98, 10863-10868.

Xu X, Yang D, Wyss-Coray T, Yan J, Gan L, Sun Y, Mucke L. 1999. Wild-type but not Alzheimer-mutant amyloid precursor protein confers resistance against p53-mediated apoptosis. Proc Natl Acad Sci USA 96: 7547–7552.

Xu X, Shi YC, Gao W, Mao G, Zhao G, Agrawal S, Chisolm GM, Sui D, Cui MZ. The novel presenilin-1-associated protein is a proapoptotic mitochondrial protein. J Biol Chem. 2002 Dec 13;277(50):48913-22

Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brashier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrikson, R. L., and Gurney, M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer'sdisease beta-secretase activity,.Nature 402, 533-537

Yanagisawa, K. Cholesterol and Pathological Processes in Alzheimer's Disease Journal of Neuroscience Research 70:361–366 (2002)

Yang, D.S.; Tandon,A.; Chen,F.; Yu,G.; Yu,H.; Arawaka,S.; Hasegawa,H.; Duthie,M.; Schmidt,S.D.; Ramabhadran,T.V.; Nixon,R.A.; Mathews,P.M.; Gandy,S.E.; Mount,H.T.; George-Hyslop,P.; Fraser,P.E. Mature glycosylation and trafficking of nicastrin modulate its binding to presenilins(2002) *J.Biol.Chem.* **277,** 28135-28142

Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C.Omi/HtrA2 catalytic cleavage of inhibitor of apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis. Genes Dev. 2003 Jun 15; 17(12): 1487-96.

Yankner B, Duffy L, Kirschner D (1990) Neurotrophic and neurotoxic effects of amyloid ß protein. Science 259: 279-282

Yin KJ, Lee JM, Chen SD, Xu J, Hsu CY. Amyloid-beta induces Smac release via AP-1/Bim activation in cerebral endothelial cells. J Neurosci. 2002 Nov 15;22(22):9764-70.

Yu G, Nishimura M, Arawaka S, Levitan D, Zhang L, Tandon A, Song YQ, Rogaeva E, Chen F, Kawarai T et al.: Nicastrin modulates presenilin-mediated Notch/glp-1signal transduction and ßAPP processing. Nature 2000, 407:48-54.

Yuan J, Shaham S, Ledoux S, Ellis HM, Hovitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell. 1993 Nov 19;75(4):641-52.

Yuan J, Yankner BA. Apoptosis in the nervous system. Nature 2000;407:802-9.

Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J.L. Vayssiere, P.X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J. Exp. Med. 181:1661–1672.

Zerbinatti CV, Wozniak DF, Cirrito J, Cam JA, Osaka H, Bales KR, Zhuo M, Paul SM, Holtzman DM, Bu G.Increased soluble amyloid-{beta} peptide and memory deficits in amyloid model mice overexpressing the low-density lipoprotein receptor-related protein. Proc Natl Acad Sci U S A. 2004 Jan 19

Zhang S, Liu J, Dragunow M, Cooper GJ. Fibrillogenic amylin evokes islet beta-cell apoptosis through linked activation of a caspase cascade and JNK1. J Biol Chem. 2003 Dec 26;278(52):52810-9.

Zhang XD, Gillespie SK, Hersey P. Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways. Mol Cancer Ther. 2004 Feb;3(2):187-97.

Zhang Y, McLaughlin R, Goodyer C, LeBlanc A: Selective cytotoxicity of intracellular amyloidbeta peptide1–42 through p53 and Bax in cultured primary human neurons. J Cell Biol 2002;156:519–529.

Zhao,B.; Chrest,F.J.; Horton,W.E.,Jr.; Sisodia,S.S.; Kusiak,J.W. Expression of mutant amyloid precursor proteins induces apoptosis in PC12 cells (1997) *J.Neurosci.Res.* **47,** 253-263

Zheng H, Jiang M, Trumbauer Me, Sirinathsinghji Djs, Hopkins R, Smith Dw, Heavesn Rp, Dawson Gr, Boyce S, Conner Mw, Stevens Ka, Slunt Hh, Sisodia Ss, Chen Hy, And Van Der Ploeg Lht. b-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81: 525–531, 1995.

Zhivotovsky B, Samali A, Gahm A, Orrenius S., Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ. 1999 Jul;6(7):644-51.

Zhu X, Raina AK, Rottkamp CA, Aliev G, Perry G, Boux H, Smith MA. Activation and redistribution of c-jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. J Neurochem. 2001 Jan; 76(2): 435-41.

8. Abbreviations:

a2M - a2-Macroglobulin

DR4 and DR5 - death receptors 4 and 5

ACE - Angiotensin converting enzyme

AD - Alzheimer's disease

AICD - APP intracellular domain: C57/C59

APAF-1 - Apoptotic protease activating factor-1

Apo E - Apolipoprotein E

APP - Amyloid precursor protein

Aß - beta amyloid peptide

ATP - adenosine-triphosphate

BACE1 - b-site APP cleavage enzyme

BSA - bovine serum albumin

CAD - caspase activated desoxyribonuclease

cAMP - cyclic adenosine-monophosphate

CARD - caspase recruitment domains

CDK5 - cyclin-dependent kinase-5

cDNA - complementary deoxyribonucleic

CED - cell death abnormal

CNS - Central Nervous Sytem

CTF - C-terminal fragment

DD - death domain

DED - death effector domains

DISC - death-inducing signalling complex

DMSO - Dimethyl-sulfoxide

DNA - Deoxyribonucleic acid

DNA-PK - DNA-dependent serine/threonine kinase

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

ER - Endoplasmatic Reticulum

FACS - Fluorescence-activated cell sorter

FAD - familial forms of early-onset Alzheimer's disease

FADH2 - Flavine adenine dinucleotide

FCS - Fetal calf serum

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GPx - Glutathione peroxidase

GSK3ß - Glycogen synthase kinase-3

HS -Horse Serum

IAP - endogenous caspase inhibitors

IDE - Insulin degrading enzyme

IgG - immunoglobulin G

JIP-1 - c-Jun NH2 terminal kinase interacting protein-1

JNK - c-Jun N-terminal kinase

LRP - Low density receptor-related Lipoprotein

MAPK - Mitogen-activated protein kinase

NADH - Nicotinamide adeninedinucleotide

NFT - Neurofibirllary tangles

NICD - Notch intracellular domain

NO - Nitric oxide

NOS - Nitric oxide synthase

NTF - N-terminal fragment

PA - polyacrylamide

PAGE - polyacrylamide gel electrophoresis

PARP - Poly(ADP-ribose) polymerase

PBS - phosphate buffered saline

PBS - Phosphate buffered saline

PMSF - phenylmethylsulfonylfluoride

PS1 - Presenilin 1

PS2 - Presenilin 2

PSAP -Novel presenilin-associated protein

RIP - Receptor-interacting protein

RNA - ribonucleic acid

RT - Reverse Transcriptase

RT - room temperature

SAPK - Stress-activated protein kinase

SDS - sodium dodecyl sulphate

SOD - Superoxide dismutase

TACE - Tumor necrosis factor-a converting enzyme

TBS - Tris-buffered saline

TM - Transmembrane

TNFR1 - Tumor necrosis factor receptor 1

TRADD - TNF receptor-associated death domain

TRAIL - TNF-related apoptosis-inducing ligand

Amino acids:

Alanine	ala	A
Arginine	arg	R
Aspararagine	asn	N
Aspartic acid	asp	D
Cysteine	cys	C
Glutamine	gln,	Q
Glutamic acid	glu	E
Gycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucin	leu	L
Lysin	lis	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	val	V

9. Bibliography:

Original Publications:

C.A. Marques, K. Schindowski, N. Touchet, C. Czech, W.E. Müller, A. Eckert. "The role of Caspase 3 cleaved C-terminal Fragment of Alzheimer's Disease related Presenilin-1 in Neuronal Cell Death", *in prep*

U. Keil, **C.A. Marques**, A. Bonert, I. Scherping, J. Weyermann, J. Strosznajder, B. Steiner, C. Haass, W.E. Müller, A. Eckert "Involvement of NO in Aß induced mitochondrial dysfuction", *submitted*

C.A. Marques, U. Keil, A. Bonert, B. Steiner, C. Haass, and W.E. Müller, Eckert A. "Neurotoxic Mechanisms Caused by the Alzheimer's Disease-linked Swedish APP Mutation: Oxidative stress, Caspases and JNK Pathway"
Journal of Biological Chemistry 278 (30): 28294-28302 Jul 25 2003

A. Eckert, **C.A. Marques** , U. Keil , K. Schüssel , W.E. Müller "Increased apoptotic cell death in sporadic and genetic Alzheimer's disease". *Ann. N.Y.Acad. Sci.* (2003) 1010:1-6

A. Eckert , U. Keil , **C.A. Marques**, A. Bonert , C. Frey , K. Schüssel, W.E. Müller "Mitochondrial dysfunction, apoptotic cell death, and Alzheimer´s disease" *Biochem Pharmacol,* 66 (8): 1627-1634 Oct 15 2003

S. Leutz, B. Steiner, **C.A. Marques**, C. Haass, W.E. Müller, A. Eckert "Reduction of trophic support enhances apoptosis in PC12 cells expressing Alzheimer's APP mutation and sensitizes cells to staurosporine-induced cell death ", *J Mol Neuroscience* 18 (3): 189-201 Jun 2002

A. Eckert, B. Steiner, **C. Marques**, S. Leutz , H. Romig, C. Haass, W.E. Müller "Elevated vulnerability to oxidative stress-induced cell death and activation of caspase-3 by the Swedish amyloid precursor protein mutation", *J Neuroscience Res* 64 (2): 183-192 APR 15 2001

Abstracts:

Keil U, **Marques CA**, Haass C, Muller WE, Eckert A Nitric oxide-induced mitochondrial failure in Alzheimer's disease N-S ARCH PHARMACOL 369: 332 Suppl. 1 MAR 2004

Bonert A, **Marques CA**, Keil U, Muller WE, Eckert A The impact of mutant APP on members of the BCL-2 family and on the release of mitochondrial factors in oxidative stress-induced cell death N-S ARCH PHARMACOL 369: 61 Suppl. 1 MAR 2004

Marques CA, Steiner B, Bonert A, Müller WE, Eckert A. Intracellular signal cascade triggered by beta amyloid 5th Int Conference on Cell and Molecular Biology of Alzheimer's disease, Hamburg, Germany , Nov 2003

Eckert A, **Marques CA**, Bonert A, Keil U, Steiner B, Haass C, Müller WE. Neurotoxic mechanism caused by the Alzheimer's disease-linked Swedish APP mutation: oxidative stress, caspases and JNK pathway 33rd Annual Meeting Soc. Neuroscience, New Orleans, USA, 2003.Soc. Neurosci., Abstracts 29, 877, 2003

Keil U, **Marques CA**, Bonert A, Strosznajder JB, Müller WE, Eckert A. Nitric oxide-induced mitochondrial dysfunction in Alzheimer's disease Pol. J. Pharmacol., 2003, 55 (5), 828

Eckert A, **Marques CA**, Keil U, Bonert A, Steiner B Muller WE Müller WE. Neurotoxic mechanisms by the Alzheimer's disease-linked Swedish APP mutation: Mitochondrial dysfunction, caspases and JNK pathway N-S ARCH PHARMACOL 367: 324 Suppl. 1 MAR 2003

Marques CA, Keil U, Bonert A, Steiner B, Haass C, Muller WE, Eckert A. Neurotoxic mechanisms triggered by 9-amyloid: Role of oxidative stress, caspases and JNK pathway Pharmacopsychiatry 36 (5): 183 SEP 2003

Marques CA, Steiner B, Müller WE, Anne Eckert. Apoptotic cell death mechanisms in familial Alzheimer's Disease- Apoptosis 2003 Meeting: From signaling pathways to therapeutic tools, Luxemburg 2003 POSTER XVII, 28.

Marques CA, Keil U, Steiner B, Haass C, Müller W.E, Eckert A. The swedish APP-Mutation leads to enhanced activation of different cell death mechanism in response to oxidative stress 32nd Annual Meeting Soc. Neuroscience, Orlando, USA, 2002. Soc. Neurosci., Abstracts 193.7, 2002.

Marques CA, Steiner B, Haass C, Müller WE, Eckert A. Activation of different cell death mechanism by the swedish APP-Mutation leads to enhanced activation in response to oxidative stress 4th Int Conference on Cell and Molecular Biology of Alzheimer's disease, Hamburg, Germany ,Sept 2002, Poster 15

Eckert A, **Marques CA**, Steiner B, Müller WE. Oxidative stress enhances activation of caspases and c-jun N-terminal kinase pathway in PC12 cells expressing Alzheimer's APP mutation NEUROBIOLOGY OF AGING 23 (1): 1862 Suppl. 1 JUL-AUG 2002

Marques CA, Steiner B, Haass C, Müller WE, Eckert A. Increased caspase-2,-3 and-8 activation in PC12 cells expressing Alzheimer mutations after exposure to oxidative stress 43. Frühjahrstagung der DGPT, Mainz, Germany, 2002 N-S ARCH PHARMACOL 365: 328 Suppl. 1 MAR 2002

Eckert A, Leutz S, **Marques CA**, Steiner B, Haass C, Müller WE. Reduction of trophic support increases the susceptibility to apoptosis in PC12 cells expressing Alzheimer's APP mutation 43. Frühjahrstagung der DGPT, Mainz, Germany, 2002 N-S ARCH PHARMACOL 365: 329 Suppl. 1 MAR 2002

Marques CA, Steiner B, Haass C, Müller WE, Eckert A. Involvement of caspases in oxidative stress-induced cell death in PC12 cells bearing Alzheimer mutations 22. Symposium der AGNP, Nürnberg, Germany 2001 Pharmacopsychiatry, abstracts, 133, 2001

Marques CA, Steiner B, Haass C, Müller WE, Eckert A. The role of caspases in oxidative stress induced cell death by ß-Amyloid in Alzheimer disease German Graduate Student Meeting, Frankfurt, Germany, 2002 Arch. Pharm. Med. Chem, 15, 2002

Marques CA, Eckert A, Steiner B, Haass C, Müller WE. Activation of caspase-3 by the Swedish amyloid precursor protein mutation 42. Frühjahrstagung der DGPT, Mainz, Germany, 2001 N-S ARCH PHARMACOL 363 (4): 348 Suppl. S APR 2001

Eckert A, Leutz S, **Marques CA**, Steiner B, Haas C, Müller WE. Elevated vulnerability to oxidative stress-induced cell death and activation of caspase-3 by the Swedish amyloid precursor protein mutation 31st Annual Meeting Soc. Neuroscience, San Diego, USA, 2001 Soc. Neurosci., Abstracts 28,547, 2001

Schindowski K, **Marques C**, Leutner S, Eckert A. Müller WE, Anti-apoptotic action of C-terminal fragment of Alzheimer's disease Presenilin 1. 30th Annual Meeting Soc. Neuroscience, New Orleans, USA, 2000 Soc. Neurosci., Abstracts 26, 474.6, p.1281, 2000

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